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PHYSICAL METHODS OF STERILIZATION OF MICROÖRGANISMS

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CONTENTS

The means by which man fights microörganisms are ordinarily classified either as chemical means (commonly called disinfectants or antiseptics), or as physical means which include a number of very different agencies, such as heat, drying, grinding, pressure and others. While the contrast between the two types of agencies as such is sharp and fundamental, certain physical causes, e.g., radiations, may bring about chemical changes, and the ultimate cause of death may be a chemical reaction although brought about by a physical agent. Plainly physical even in its ultimate analysis is death by mechanical destruction, as by grinding. Plainly chemical is the slow death of dry bacteria which is due to oxidation of some essential cell constituents and follows the laws of chemical disinfection. Between the two extremes stand heat and radiation which destroy life by denaturation of some important cell proteins. This denaturation may be considered a chemical or a physical process.

Facts can be used to greatest advantage when the reasons for the facts are completely understood. The object of this review is not an enumeration of facts, but an attempt to correlate the knowledge acquired about physical disinfection, and to understand how physical agents can kill bacteria, or, more generally, how they can bring about the death of any cell.

The applications in the home, in industry and medicine, although of widest use and inestimable value, cannot be considered here. Drying and freezing have been used since prehistoric times to preserve food, but these processes do not sterilize. They may be compared with antiseptics rather than with disinfectants, because they prevent bacterial action and may kill a large proportion of microörganisms, but cannot be relied upon to kill all of them. The use

of artificial ultraviolet light, to destroy bacteria is of rather recent date But the application of heat antedates Pasteur's discovery that food spoilage is caused by microörganisms Appert based his process of preserving foods by long-continued heating upon the theory that the air over the food in the container was "rendered to no effect by the action of heat" A considerable canning industry had developed long before Pasteur published his first papers

I MECHANICAL CAUSES OF DEATH

1 Death by grinding and shaking It is obvious that cells are dead when they are broken into many small pieces Experiments with protozoa have shown that recovery is possible only when the nucleus has remained uninjured With bacteria and yeasts, recovery after mechanical injury may also be expected if the damage is slight

To what extent the smaller microörganisms might be broken up mechanically, was not known until Buchner's famous demonstration of cell-free fermentation (1897) Yeast ground with quartz sand was still capable of changing sugar to alcohol and carbon dioxide, but had lost the power to produce colonies on nutrient agar This experiment calls attention to the bacteriologist's definition of death which differs from the definitions by all other biologists A bacterium (or yeast) is considered dead when it has lost the power to reproduce

Bacteria can also be killed by shaking, but vigorous agitation is necessary to bring about a noticeable decrease in the number of viable cells (Campbell Renton, 1942b)

An interesting combination of grinding and shaking has been studied by Curran and Evans (1942) who shook bacterial cultures or spore suspensions with different kinds of abrasives, such as glass beads, sand, or carborundum With uniform shaking on a mechanical shaker, the number of fatal hits per minute must be directly proportional to the number of cells present With n bacteria at the start, and the fraction p killed per hour (or $100p$ % per hour), the number of dead bacteria after the first hour of shaking is np, and the number of survivors is $n(1-p)$ Of these survivors, the fraction p dies again during the next hour, and the fraction $(1-p)$ survives, which makes the survivors after 2 hours of shaking $n(1-p)\,(1-p)$ After the third hour, the survivors number $n(1-p)^3$ and after t hours, $n(1-p)^t$ If we call the number of survivors b, we have

$$b = n(1-p)^t$$

$$\frac{b}{n} = (1-p)^t$$

$$\log b - \log n = t \log(1-p)$$

$$-t \log (1-p) = \log n - \log b \qquad \text{(a)}$$

In this equation, p is a constant, and therefore also $\log (1-p)$, and we may substitute $-\log (1-p) = K$ This simplifies equation (a) to

$$Kt = \log n - \log b = \log \frac{n}{b}$$

where n is the original number of bacteria, b the number of survivors after t hours of shaking, and K a constant measuring the rate of death[1] According to this equation, the logarithms of survivors plotted against time must fall on a straight line As figure 1 shows, this is actually the case

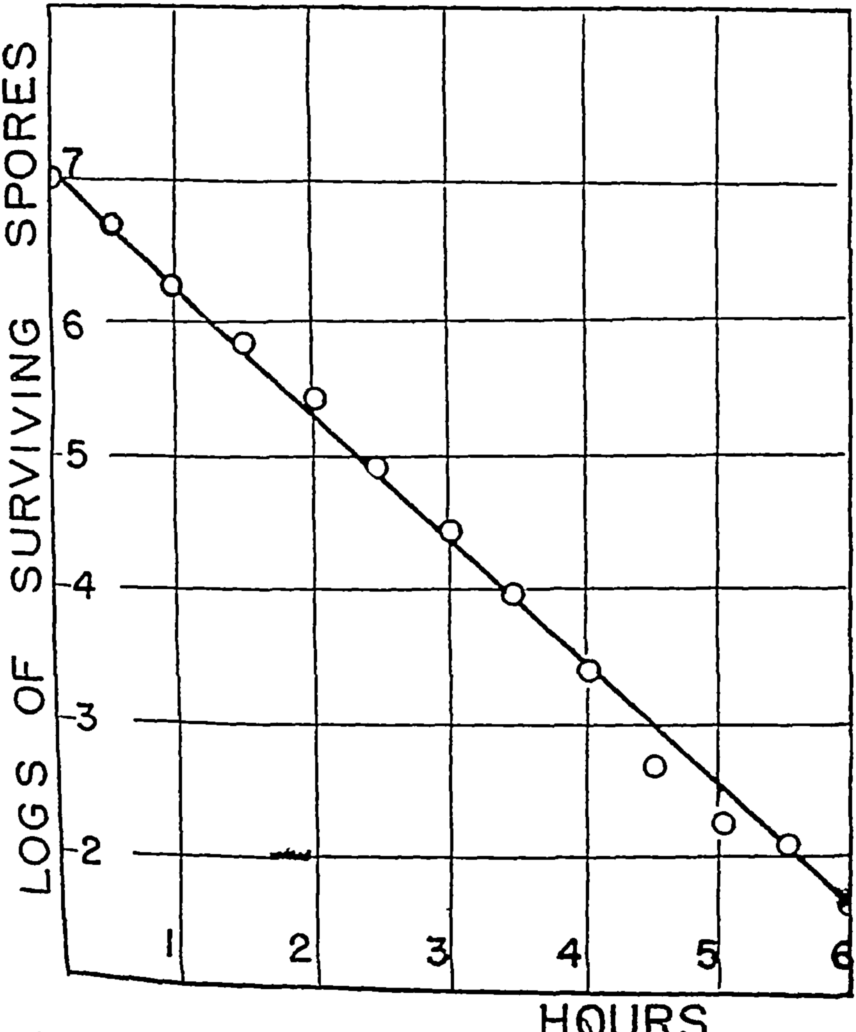

FIG 1 SURVIVOR CURVE OF THE SPORES OF BACILLUS SUBTILIS SHAKEN WITH GLASS BEADS (FROM CURRAN AND EVANS, 1942)

The equation is identical with that claimed for the logarithmic order of death by disinfectants or heat Such identity does not indicate any relation between the two causes of death They are so entirely different that no conclusion can be drawn from one to the other In the shaking experiments, the order of death

[1] The percentage of cells killed per hour (100 p) can be computed as follows

$$\log (1-p) = -K$$
$$1-p = 10^{-K}$$
$$p = 1 - \frac{1}{10^K}$$

can be predicted, it must be logarithmic, and experiment has proved the reasoning to be correct With chemical disinfection, this type of order was entirely unexpected, and its explanation and even its existence has been debated for more than 30 years The equation has been considered in some detail here because radiation, according to the corpuscular theory of light, may be considered as a bombardment with electrons or photons or quanta, and this must result in a logarithmic order of death which will be discussed in a later chapter

Table 1 shows some of the data obtained by Curran and Evans The chemical nature of the abrasive, its degree of hardness and its specific gravity had no influence on the death rate Spherical particles appeared more efficient than angular ones, perhaps because they produced larger surfaces of contact by sliding or rolling past each other The particle size was of great influence as may be seen from the table Sand was found most efficient when the grain size was between sieve numbers 40 and 60, while glass beads destroyed bacteria most rapidly when they passed sieve No 60, but not 80 An increase in size resulted in a lower death rate, and a decrease had the same effect

TABLE 1

Destruction of spores suspended in buffer solution (pH 7 0) when shaken with abrasives for 5 hours at 480 rpm

ABRASIVE (20 g IN 25 ml OF BUFFER)		SPORES OF BACILLUS COHAERENS			SPORES OF BACILLUS MEGATHERIUM		
		Survivors per ml	Death rate K (per hour)	Per cent killed per hour	Survivors per ml	Death rate K (per hour)	Per cent killed per hour
At start		1,350,000	—	—	950,000	—	—
Particles passed No 20 sieve but not No 40	Sand	70,000	0 293	49 1	7,300	0 423	62 2
	Pyrex chips	39,800	0 342	54 5	750	0 620	76 0
	Carborundum	95,500	0 231	41 2	8,050	0 414	61 4
	Boron carbide	44,000	0 297	49 5	5,400	0 449	64 4
	Glass beads	42,800	0 301	50 0	2,700	0 509	69 0
Particles passed No 80 sieve but not No 100	Alundum	172,000	0 179	33 8	30,200	0 300	49 8
	Emery	173,000	0 178	33 6	20,800	0 332	53 4
	Carborundum	7 900	0 446	64 2	300	0 700	80 1
	Boron carbide	120,000	0 210	38 3	17,400	0 347	55 1
	Glass beads	30	0 931	88 3	3	1 100	92 1

Vegetative cells were more sensitive than spores, but the difference was not great With the spores of *Bacillus cohaerens*, when shaken with glass beads in distilled water, the death rate constant was $K = 0.8$ *Escherichia coli* under identical conditions had the constant 1.8 The same *E coli* shaken with the same glass beads in broth showed a death rate constant of only 0.8, the protection being probably due to the foaming The spores of different species differ considerably in their sensitivity to mechanical destruction, as table I shows, and this difference is not correlated with the difference in heat resistance

According to Campbell-Renton (1942b), bacteriophage is sensitive to shaking, but a great variation of sensitivity was observed with different phages The shaking was carried out without addition of solid particles With *Salmonella schottmuelleri*, the phage was inactivated to a much greater extent than the bacteria It is possible to obtain apparently phage-free cultures of bacteria by shaking, provided that the culture is not too heavily infected with phage

2 *Death by pressure* It is not very probable, reasoning *a priori*, that pressure can affect bacteria suspended in a liquid As there are no gas-filled spaces in the cell, the change in pressure can result only in slight differences in volume and the cells are not likely to be torn, unless the change is very sudden as with supersonic waves Pressure cannot change the cell constituents greatly, nor can it alter their relative position The theory of Johnson, Eyring and Williams (1942) that pressure changes the equilibrium between native and denatured protein does not apply in this case because in death, we are dealing with an irreversible process

Chlopin and Tammann (1903) placed 24-hour broth cultures of many different microörganisms in sterile castor oil which was then subjected to pressures up to 2900 atmospheres, the pressure being increased in steps of 500 atmospheres every 15 minutes A pressure of 2000 atmospheres for 4 hours at 36° killed the entire cultures of *Eberthella typhosa*, *Salmonella typhimurium*, *Vibrio cholerae* and *Micrococcus agilis*, *i e*, they had lost the power to reproduce, but the cells were still motile Other species were weakened, but some cells survived Virulence was considerably decreased, and remained decreased An exposure to 2000 atmospheres for 4 days at 14–16° killed most of the species tested, but not *Bacillus anthracis*, *Oidium lactis*, *Corynebacterium pseudodiphthericum*, and beer yeast All of these species were greatly weakened Again, many of the "dead" bacteria remained motile The increase to 2900 atmospheres did not change the result greatly Rapid increase and decrease of pressure had little influence

In 1914, Hite and associates attempted to preserve milk, vegetables and fruits by pressure in place of the customary application of heat in canning They found that 100,000 pounds per square inch, at room temperature for seven days, did not destroy some of the milk enzymes, but no culture of bacteria could be obtained from such samples "In old milk, an original count of 30 or 40 million bacteria per cc may be cut down to a few hundred, or a few dozen, by an application of 100,000 pounds for 10 minutes"

Grape juice, cider, peaches and pears could be sterilized by 60,000 pounds pressure in 30 minutes, while blackberries and raspberries usually fermented after such treatment Vegetables spoiled almost universally, and tomatoes began to sour after having been exposed to such pressure

Experiments with pure cultures of different species showed certain differences The logarithms of the death times plotted against the logarithms of the pressures fell on a straight line in most cases (fig 2) Pressures less than 30,000 pounds or 2,000 atmospheres did not kill pure cultures in 3 hours This agrees fairly well with Tammann's findings

A different picture is obtained when the cultures are exposed to gas pressures, because this causes a chemical change of the environment Compressing the air above the culture to 100 atmospheres would mean approximately 100 times as much dissolved oxygen in the medium, and this might kill even many aerobic species The effect of such compressed gases is really chemical disinfection It becomes physical only when the sudden release of the gas pressure tears the cell This latter possibility will be treated at the end of this chapter

Oxygen That high concentrations of oxygen are toxic to bacteria, has been known for a very long time In his review on inhibition of bacteria by oxygen, Rodenkirchen (1937) mentions experiments as early as 1873 Precise experiments with measured oxygen pressures and pure cultures were made by Porodko

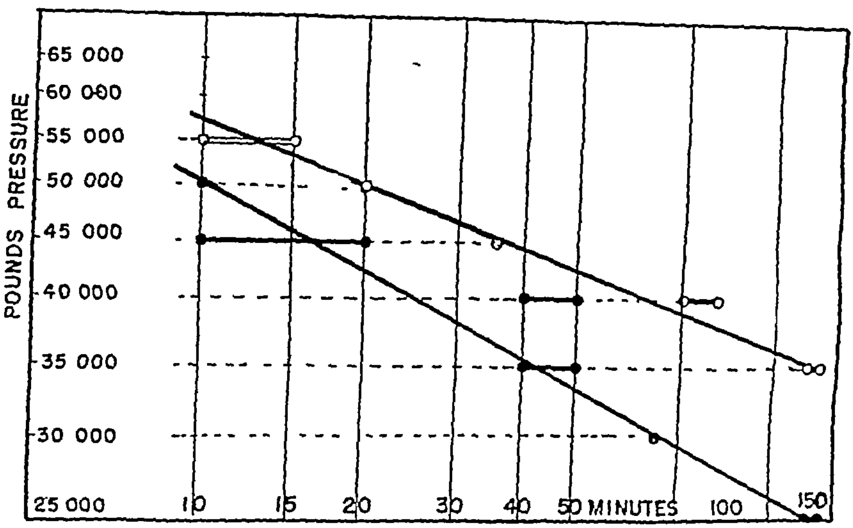

FIG 2 THE DOUBLE LOGARITHMIC RELATION BETWEEN PRESSURE AND DEATH TIME ABOVE SERRATIA MARCESCENS BELOW SACCHAROMYCES CEREVISIAE (DATA OF HITE, GIDDINGS AND WEAKLY, 1914)

(1905) and Berghaus (1907) Inhibition of growth for 4 days was obtained by 2 atmospheres of pure oxygen with a pink yeast, *Bacterium cyanogenum*, *Bacterium bruneum*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Bacillus anthracis*, but they were not killed completely, and they multiplied when the oxygen pressure was released Other species like *Pseudomonas fluorescens*, *Bacillus mycoides*, *Proteus vulgaris* and *Eberthella typhosa* were inhibited by 2 5 atmospheres of oxygen, *Escherichia coli* and *Salmonella enteritidis* required 3 atmospheres, *Sarcina lutea*, *Vibrio albensis*, *Bacillus subtilis*, and *Staphylococcus aureus* 4 atmospheres, and another strain of *E coli* as well as *Serratia marcescens* 6 3 atmospheres One species of micrococcus required about 10 atmospheres

The oxygen concentrations for complete sterilization are much higher Berghaus found *B anthracis*, *Alcaligenes faecalis* and *Vibrio cholerae* dead after 21 hours' exposure to 2–4 atmospheres of oxygen pressure, but 10 representatives of

the colon-typhoid group, as well as *Pseudomonas aeruginosa* and *Staphylococcus aureus* required 60 to 75 atmospheres

This inhibition of bacterial development by oxygen has been used in the Hofius process of milk preservation which consists in keeping the milk under 8 atmospheres of oxygen pressure (120 pounds) at low temperature Such milk is claimed to keep 2 to 3 weeks (Müller, 1936, Rodenkirchen, 1937)

Hydrogen Very little effect of hydrogen pressure was observed by Larson, Hartzell and Diehl (1918) A pressure of 120 atmospheres never produced sterile cultures Of *E coli*, only 10 to 40% of the cells had been killed in 24 hours, and the microscope showed many cells to be broken up Unexpected was the result that "gram-positive bacteria would often become gram-negative, and even the acid-fast character of the tubercle bacteria was impaired "

Nitrogen at 120 atmospheres pressure did not kill the bacteria, nor did it change their morphological characters

Carbon dioxide has been tested by many authors Larson *et al* (1918) could kill non-sporulating bacteria by 50 atmospheres in about 1½ hours, but 40 atmospheres had no effect whatever Death was not due to the low pH of 3 15 because the bacteria could tolerate this acidity for 48 hours when it was produced by other acids, without the pressure of CO_2 Yeast cells survived the same pressure treatment for 48 hours These authors consider the death to be due to the "sudden change of osmotic tension "

"Gram-negative bacilli could be brought to a marked degree of disintegration, although disintegration of all the bacteria in suspension was never attained The gram-positive cocci suffered little morphologic change aside from slight irregularity in size, and often a tendency to lose their gram-positive character " Very little disintegration was noticed when the bacteria were suspended in broth or saline instead of distilled water

Swearingen and Lewis (1933) assumed that the death of bacteria under high CO_2 pressure was due to the formation of gas bubbles within the cell which would make the cell explode when pressure was released According to their calculations, about 40 pounds (2 6 atmospheres) of surface tension pressure must be overcome to form a gas bubble of the size of a bacterium Therefore, death by explosion could not occur with low CO_2 pressures However, with pressures less than 40 pounds, they observed a slow rate of death which they ascribed to the precipitation of certain colloid systems

The pressures used in carbonated drinks are not sufficient to produce sterility, even after several months Milk under 60 pounds of CO_2 will sour slowly The results of attempts to use CO_2 in food preservation are summarized in McCulloch's book (1936)

8 Death by sonic and supersonic waves Death by such waves is fundamentally not different from death by mechanical agitation It is generally assumed that the waves produce a very rapid succession of compressions and releases of the liquid, which tear the suspended cells to pieces It has been suggested that the rarefaction during release might go so far as to produce microscopically small areas of vacuum While bacteria can withstand slowly rising or decreasing

pressures, the very rapid alternation injures them The death rate rises with the frequency of the waves, which indicates that the suddenness of change between compression and release is an important factor

Harvey and Loomis (1929) proved that luminescent bacteria could be killed by supersonic waves of approximately 375,000 cycles per second By exposure for one hour or longer, complete sterility could sometimes be obtained Williams and Gaines (1930) treated cells of *Escherichia coli* with waves of only 8,800 cycles, and observed a very slow decrease of about 60 to 70% of all cells per hour The order of death was logarithmic Chambers and Gaines (1932) found a logarithmic order for *E coli* only with young cultures The 5-day culture contained many cells of greater resistance, and their percentage as well as their resistance increased up to 14 days *Streptococcus lactis* showed a strictly logarithmic order even with an 8-day culture

More extensive were the experiments by Beckwith and Weaver (1936) who used equipment similar to that of Harvey and Loomis, i e, one that yielded frequencies of about 400,000 c p s With such high frequencies, heat is generated by the waves, and the cultures under test must be cooled Aqueous suspensions of cells from a 24-hour culture of *E coli* were always sterilized by a 5-minute exposure to these ultrasonic waves while a 6-hour culture of *Eberthella typhosa* in broth had 1 to 10% survivors after 10 minutes' treatment Spores of *Bacillus subtilis* suspended in water, after 15 minutes, had decreased only about 50% With the spores of thermophilic bacteria, 98 to 99% were killed when exposed in 5% sucrose solution, and only 75% died in 20% sucrose solution Yeast in grapejuice varied enormously, sometimes 3 minutes sufficed to sterilize the culture, at other times, a few cells survived even after 15 minutes

Equally fluctuating results were obtained with the mixed flora of milk The efficiency of the method varied from 20% to 99% in terms of fatality Systematic experiments showed that the great protection exerted by milk was due not to the fat or the lactose, but to the casein The great retardation of death in the presence of proteins has spoiled all hopes that this would be an efficient method of sterilizing milk and other foods without heat

This method of disintegrating cells can be used to obtain certain cell constituents from cell suspensions because heat-labile proteins are not denatured by sound waves Chambers and Flosdorf (1936) produced cell-free antigens of *E typhosa* and *Streptococcus hemolyticus* in this way

II DEATH BY IRRADIATION

Of the wide range of radiations existing on earth, only two regions have a pronounced lethal effect on bacteria, namely, the ultraviolet range and the cathode to X-ray range Visible rays do not affect bacteria appreciably There is a possibility of a very slight effect according to Duggar (1936, p 1127)

Radiations can produce chemical or physical changes only when they are absorbed That is the reason why X-rays which have the power to penetrate organic tissues without being absorbed are not used in practical disinfection while ultraviolet rays, which are noticeably absorbed even by such transparent substances as glass and water, are applied in many ways for sterilization

Color is produced by the absorption of selected wave lengths of visible light. The color of ultraviolet light is invisible to us, but its absorption by chemical compounds can be measured quantitatively by the absorption spectrum. Gates (1934) has shown that "the destruction spectrum of pepsin by ultraviolet agrees essentially with its absorption spectrum." This verifies the above statement that only the absorbed rays can produce chemical changes.

Details of the effects induced by different radiations can be more readily interpreted after a discussion of the fundamental cause of death by such rays.

1. *The cause of death* by irradiation is the inactivation of some essential cell constituents by the energy of the absorbed rays. X-rays have been used for many years to produce mutations in plants and animals (see review by Duggar, 1936). It is assumed that a quantum absorbed by a chromosome either destroys one or several genes, or disturbs their arrangement. If the destroyed gene is essential for multiplication, the cell may remain alive, but cannot reproduce, it becomes sterile. Microörganisms are no exception. Mutations by means of X-rays have been produced in bacteria, (Haberman and Ellsworth, 1939, Lincoln and Gowen, 1942) in yeasts, (Oster, 1934, Lacassagne *et al.*, 1939) and in molds (Beadle and Tatum, 1941–2). Mutations of molds (Stevens, 1930) and of bacteria (Sharp, 1940) have also been produced by ultraviolet light, and death by ultraviolet may well be considered to be a lethal mutation. Lea and Haines (1940) used this very term, apparently without knowledge of Rahn's (1929, 1934) explanations of death and Jordan's (1940) identical definition.

The corpuscular theory of light assumes that rays are minute energy projectiles moving with an enormous speed. They differ greatly in their energy content which is released upon absorption and causes changes which may lead to death. The physicist frequently speaks of a "quantum hit" when he means absorption of a quantum. Therefore, death by radiation is death by bombardment and comparable to death by shaking with glass beads, and we must expect a strictly logarithmic order of death. Most data with cathode rays (Wyckoff and Rivers, 1930), X-rays (Wyckoff, 1930) and ultraviolet (Wyckoff, 1932, Sharp, 1939) show this. Of the survivor curves for 10 bacteria given by Sharp (1939) seven are straight, while three are concave downwards resembling the survivor curves of multicellular organisms. The exceptions are represented by two staphylococci (which form clumps) and by the thread-forming anthrax bacillus. These bacteria would be expected to produce exceptional curves (Rahn, 1930). The survivor curves of mold spores are concave downwards (Whelden *et al.*, 1940, Zahl *et al.*, 1939). This is typical for mold spores by all causes of death (Rahn, 1943). The data of Gates (1929) for staphylococci are also concave downwards. The one unexplained exception is a curve of the same shape for *E. coli* suspended in air, observed by Sharp (1940).

The simplest case is the death by cathode rays which can be considered as a bombardment with electrons. According to Wyckoff and Rivers (1930), "for the two motile bacilli, *B. coli* and *B. aertrycke*, the absorption of a single 155 K.V. electron is sufficient to cause death. The same is undoubtedly true of *Staphylococcus aureus*. Furthermore, all, or nearly all, the electrons absorbed

are lethal The differences in sensitivity to cathode rays shown by the bacteria studied can be explained by the purely physical factor of size "

The explanation is to be sought in the great energy contained in the electron The same authors state that "a 150 K V electron will liberate about 10^4 ions within less than 0 001 mm³ Together with this ionic shower, X-rays are emitted as a consequence of electron absorption " Since 0 001 mm³ is the same as 1 cubic micron, and approximately the volume of an average bacterium, one absorbed electron can ionize the entire cell to complete destruction The energy per quantum decreases as the wave length increases With X-rays, Holweck (1929) and Lacassagne (1928) observed that a cell of *Pseudomonas aeruginosa* can be killed by absorption of a single quantum, but only if it is absorbed by a definite part of the cell which they call the "sensitive zone " Wyckoff (1930) measured the average number of quantum hits required to kill a cell with X-rays

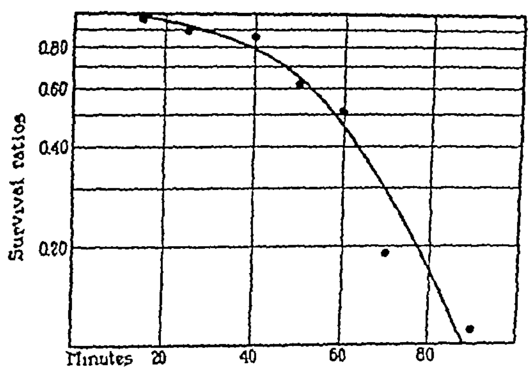

FIG 3 THE SURVIVAL RATIOS OF THE SPORES OF RHIZOPUS NIGRICANS UNDER THE ACTION OF Kα X RAYS (FROM LUYET, 1932)

of different wave lengths From these, the size of the sensitive zone could be estimated The results were

Wave length, A	0 56	0 71	1 5	2 3	4 0
Quanta required to kill	4 54	6 46	14 2	29 5	69 7
Sensitive volume (cell = unit)	0 22	0 16	0 07	0 034	0 014

Yeast cells are larger, their volume is of the order of magnitude of 100 μ^3, and it cannot be expected that every electron hit will strike a vital cell constituent Consequently several quanta are needed to kill a yeast cell (Wyckoff and Luyet, 1931) Mold spores are larger than yeast cells, and the logarithmic survivor curve of the spores of *Rhizopus nigricans*, figure 3, shows plainly that many quantum hits are necessary to kill the spore

In the case of ultraviolet radiation, several million quanta must be absorbed before a cell is killed, and Wyckoff's (1932) calculation of the "sensitive zone" proved it to be only about the size of a protein molecule Wyckoff believed that to be impossible, and considered death by ultraviolet to be quite different from that by other rays But Gowen (cf Duggar, 1936, p 1323) estimated that the

sensitive zone in *Drosophila*, which must be hit in order to produce a mutation, is about 10^{-18} cm³, or a cube with sides of 0 01 μ This is the volume of a fairly small protein molecule Fricke and Demerec (1937) estimated the average diameter of a gene to be about 25 A = 0 0025 μ Haskins and Enzmann (1936) obtained the same value Since death of bacteria can be considered as a lethal mutation, the measurement by Wyckoff supports this viewpoint very well The energy in a single quantum of ultraviolet radiation seems just sufficient to inactivate the protein molecule which absorbs it, but not sufficient to cause further effects Thus, death occurs only when an indispensable and irreplaceable protein molecule is hit by the quantum Quanta of visible light have less energy, and cannot inactivate the protein molecule even with a direct hit, and therefore cause no death

This simple theory of death has been questioned by Rentschler *et al* (1941) who believe that "the relation between the amount of ultraviolet radiation and the per cent of bacteria killed is determined by the distribution of bacteria of different resistivity to the radiation and is not due to the probability of hitting a vital spot in a given organism by a photon " They prove quite conclusively that bacteria at the stage of rapid cell division are much more sensitive than resting bacteria, at least 5 times as sensitive according to the method of calculation used However, that does not disprove other experiments which were almost always made with resting cells A graded resistance cannot explain the logarithmic order of death as Rahn (1943) has shown

These authors claim further that the single photon-hit theory can hardly explain the fact that a sublethal dose retards the rate at which colonies develop after irradiation However, this really should be expected A photon hit, i e the absorption of a quantum of ultraviolet radiation, ionizes the immediate environment of the place of absorption If no life-important gene is destroyed, there is likely to be other injury which, although reparable, may cause considerable delay of development

Disturbances of the mechanism of cell division and growth coördination by rays have been frequently recorded Luyet (1932) estimated the amount of injury by various rays upon the spores of *Rhizopus nigricans* by measuring the average length of mycelium per spore produced within 24 hours after exposure He also observed spores which swelled to nearly 5 times their diameter, but never produced a mycelium Oster (1934) reported giant cells of yeasts and two-cell groups from 3 to 8 times the size of normal two-cell groups, after exposure to ultraviolet Gates (1933) described a loss of cell division, but continuance of growth by *E coli* after ultraviolet irradiation Some cells continued to increase in size, especially in length, but did not divide, and produced filaments, sometimes 50 to 150 μ in length, with a diameter occasionally three times normal These cells finally degenerated, or began suddenly to divide

The mechanism of cell division and coördination seems to be more sensitive than that of growth as such, i e , of organic synthesis, and this again is more sensitive than that of catabolism, of enzyme action and energy provision Yeast cells exposed to a mercury vapor lamp lost the ability to produce colonies on agar

more rapidly than the ability to ferment sugar to alcohol and carbon dioxide (Rahn and Barnes, 1933) The cells retained after 20 minutes' exposure, 1 8% of viability, 60 0% fermenting capacity, after 40 minutes' exposure, 0 7% of viability, 39 0% fermenting capacity

2 Effect of temperature A single quantum of ultraviolet rays or of rays of shorter wave length, if absorbed at a specified location in the cell, destroys that cell's capacity to reproduce An increase in temperature does not increase the energy liberated by absorption It may, however, increase slightly the radius of the ionization zone around the absorbed quantum Thereby, a slight increase in deaths may be observed at higher temperatures for such cases where the quantum hit was not close enough to the sensitive zone to cause inactivation at low temperature, but is just sufficient at the higher temperature This leads to the assumption of a temperature coefficient analogous to that of photochemical reactions, which amounts to an almost negligible increment

All measurements have confirmed this assumption Hercik (1936) reported a Q_{10} of 1 for the α-particles of Polonium For ultraviolet light, Bayne-Jones and Lingen (1923) found the value 1 15, Gates (1929) found an average of 1 1, E Smith (1935) observed with Fusarium spores between 0 and 40 C a temperature coefficient of 1 13 This is definite proof that death is not caused indirectly, e g , through formation of toxic peroxides The lethal effect of peroxides would have a much higher temperature coefficient

3 Effect of wave length The death rate depends upon the number of quanta absorbed as well as upon the energy per quantum In the range of cathode rays and X-rays, no preferential absorption of certain wave lengths has ever been observed, and death depends only upon the amount of incident energy

In the ultraviolet range, different organic compounds are characterized by their preferential absorption of certain wave lengths, and we must expect the strongly absorbed wave lengths to cause more damage per erg per mm² of incident energy than those wave lengths which are but slightly absorbed Gates (1930) determined the absorption curve for ultraviolet with *Staphylococcus aureus* and *E coli* and found important points of similarity and of difference with the bactericidal curves Ehrismann (1930) obtained essentially the same results The difference begins with wave lengths longer than 2800 A There, the great absorption is not accompanied by a corresponding death rate, probably because of the low energy per photon

In practically all species investigated by Ehrismann, Coblentz and Fulton, Duggar and Hollaender, Gates, and Wyckoff, the greatest absorption takes place around 2650 A, and at this wave length, the largest number of cells per erg of incident energy is killed With longer and with shorter wave lengths, the percentage of killed individuals decreases At 3300 A, the deaths per erg are less than 1% of that obtained near 2650 A, and at 2400 A, about 50% of this maximum (see fig 4)

In his study of the effect of the entire range of electromagnetic waves on three fungi, Johnson (1932) found that the *only* effect of the visible light rays was an increase in pigment in *Fusarium batatatis* after exposure for a week or more No

effect was observed after exposure to infra-red rays above 7,000 A, or to Hertzian waves of 50 and 100 m

4 Sensitivity of different species The differences between different species in their resistance to X-rays or cathode rays have been explained by Wyckoff (1930) to be due simply to differences in size Almost any absorbed quantum of these rays is lethal, and there seems to be no significant species difference of absorption

This is also true for ultraviolet radiation All investigations have shown that the sensitivity of different species of bacteria varies but little Sharp (1939) working with 10 species reports, as extreme variations of energy required to kill, 168 ergs per mm² for dysentery bacteria, and 337 ergs for diphtheria bacteria Even bacterial spores are easily killed Sharp found in 1939 that a culture of *B anthracis* with spores required 452 ergs In 1940, he observed that a spore-containing culture of *B subtilis* sprayed into air had to be exposed 2 to 3 times as long as *E coli* to obtain the same killing effect Duggar and Hollaender

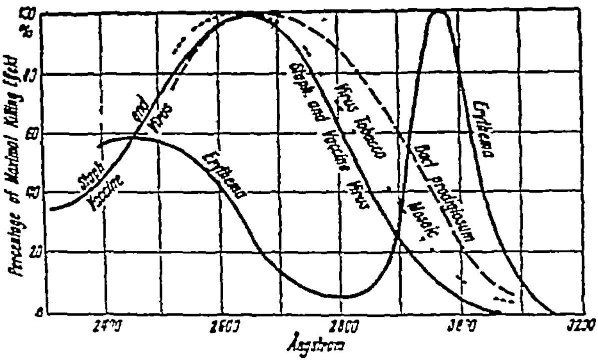

FIG 4 COMPARATIVE INTENSITIES OF THE KILLING EFFECT OF DIFFERENT WAVE-LENGTHS ACTING ON DIFFERENT ORGANISMS (FROM RAHN, 1936)

(1934) could kill 85% of the vegetative cells of *B subtilis* and *B megatherium* with 165 ergs per mm² while the spores needed 182 ergs Lea and Haines (1940) found the spores of *B mesentericus* to require 5 times as much energy as *E coli*

The similarity of the death curves suggests that the molecules, whose inactivation by ultraviolet causes death, are similar in the different species This agrees with the conception of death as a lethal mutation, because genes must be considered as nucleoproteins, and though they may differ specifically, their absorption spectra are probably quite similar

The spores of molds are more resistant, and the resistance varies greatly with the species Fulton and Coblentz (1929) studied the lethal action of ultraviolet radiation upon the spores of 27 widely different species Sixteen of these could be killed by a 1-minute exposure to a mercury-tungsten lamp, with 4 others, less than 1% survived, but the two most resistant species had between 40 and 50% survivors after a 4-minute exposure The authors explain this by "the difficulty in ray penetration of the spore walls due to their protective coloration or to their composition " The mycelium is more easily killed than

the spores The spores of *Penicillium digitatum* required 9 times as long an exposure as *E coli* This penicillium belonged to the 16 easily killed species, and the observation by Koller (1939) that spores of *Aspergillus niger* require 50 to 100 times as much energy as *E coli* is not contradictory

The virus of tobacco mosaic was far more resistant than the spores of *B subtilis* or of *B megatherium*, but responded essentially to the same wave lengths (Duggar and Hollaender, 1934)

Ultraviolet rays are widely used for the sterilization or air, especially in hospitals and operating rooms (see review by Hart and Sharp in Glasser's Medical Physics, 1944) They are also employed to decrease the contamination from the air of breweries, bakeries, meat and vegetable storage rooms (see review by Porter, 1940)

Ultraviolet radiation has been used for the sterilization of the water supplies of a few cities in France The process is efficient, but expensive Many laboratory attempts have been made to sterilize milk, but this method has not been used as yet by the dairy industry (Supplee *et al*, 1941) Sterilization of solid objects must necessarily be limited to the very surface, and while fair success is claimed for meat in storage (Porter 1940), Fulton and Coblentz obtained discouraging results in trying to sterilize oranges Hall and Keane (1939) could kill all the spores of thermophilic bacteria in sugar in laboratory experiments, but in large scale manufacturing, ultraviolet radiation destroyed only half of the spores, on account of the absorption of the rays by the sugar crystals

III DEATH DURING AND AFTER DESICCATION

Two different effects must be considered separately when the desiccation of bacteria is concerned, namely, the number of fatalities due to the removal of moisture, and the gradual death of those bacteria which survive the change from the moist to the dry state The two causes of death are quite different, and are independent of each other

1 *Death during desiccation* The earlier theories referred only to the death of bacteria spread in thin layers on some surface, and it was believed that bacteria could not survive complete drying, but were protected against this occurrence more or less completely by the capsule of the dry medium around them, and only those cells could survive which kept their natural moisture content Modern drying methods, especially the spray-drying, leave only very thin protective layers around the bacteria, and yet many survive The percentage of survivors may vary from 0 to nearly 100, depending not only upon the species and age of the culture, but upon the kind of medium in which the cells are suspended, the surface on which they are dried, and the rate and temperature of drying

Most important is the medium surrounding the bacteria during the act of desiccation Bacteria dried with their culture medium such as broth or milk survive fairly well while suspensions of bacteria from agar surfaces or of washed bacteria have only a very small percentage of survivors

Paul and Prall (1907) dried the staphylococci from agar surface growths, after

suspension in water, on small stones (garnets) of uniform size in order to have bacteria free from the organic matter of the medium A decrease of about 60% was observed in the first 24 hours, but after that, the number of survivors remained constant if they were kept in a vacuum at very low temperatures The object of Paul and Prall's procedure was to obtain uniform bacterial suspensions for testing disinfectants in the absence of organic matter However, the dead bacteria were actually merely organic matter This becomes very evident from the study by Otten (1930) who made thick suspensions of bacteria from agar surface growths in saline solutions, and dried small quantities, a few drops or ½ to 1 ml, in tiny vials at room temperature in a vacuum over concentrated H_2SO_4 He obtained a survival rate of 2 to 5% with typhoid bacteria, 0 05 to 0 005% with dysentery bacteria, and only one survivor out of 10,000, sometimes less than one out of a million, with the cholera vibrio Otten varied the conditions of drying, and observed that slow drying kills more bacteria than rapid drying Quite important was the depth of the layer of dried cells While 1 ml of a suspension of typhoid bacteria resulted in 0 04% survivors after drying, 0 1 ml of the same suspension dried on the same surface gave only 0 008% survivors The same amount of suspension, dried in different containers of which one had an exposed surface ten times as large as the other, gave survival ratios 5 to 23 times as large with the smaller exposed surface Otten then mixed the bacteria to be dried with a suspension of dead bacteria, and obtained far better survival, for instance

1 ml of the concentrated suspension yielded	2 6%	survivors
1 ml of a dilution (1 10) with saline yielded	0 08%	"
	0 03%	"
1 ml of a dilution (1 10) with a suspension of dead bacteria yielded	2 3%	"
	1 8%	"

Otten emphasizes that the bacterial proteins protect the cells not by forming a cover which prevents complete desiccation, but by acting as protective colloid which makes the drying process more gentle and less abrupt In 1933, Otten applied this discovery to the drying of very sensitive species, such as the bacteria of meningitis or whooping cough, by the addition of dead staphylococci He also showed that dried smallpox vaccine, was protected by the lymph proteins and could be kept active at tropical temperature for as long as 5 years by preservation *in vacuo* (fig 5b)

The majority of experiments on desiccation refer to bacteria dried with the culture medium which is practically always of colloidal nature Consequently, the survivor ratio is high Rogers (1914), in his first experiments, dried freshly curdled milk cultures of lactic streptococci by adding an equal amount of lactose, and blowing warm air of 43 C over the culture About 80% of the cells died during this treatment (table 2), and the death rate was greatest when the moisture dropped from 10% to 5%

Higher survival rates were obtained by spraying the cultures into a current of

dry warm air Drying was almost instantaneous, and took place at fairly low temperatures because of the rapid evaporation Initial bacterial counts are not given, but the powdered cultures contained from 657 million to 8,590 million viable cells The best method, however, was the desiccation of frozen cultures For laboratory experiments, the cultures were frozen in Petri dishes in CO_2-snow, placed in a cold desiccator with concentrated H_2SO_4, or P_2O_5, and evacuated to a very high vacuum which is absolutely essential for rapid drying 10 ml of a milk culture can thus be dried in 3 to 4 hours The frozen and dried cultures contained between 380 and 12,670 million bacteria per gram Neutralization of the milk cultures resulted in higher bacterial counts before drying, but in lower counts after drying

"The Bureau of Dairy Industry prepares, for distribution in the field, dried cultures of *Propionibacterium shermanii*, the organisms largely responsible for the characteristic flavor and eye formation in Swiss cheese The final product

TABLE 2

Survival of lactic streptococci during drying of a milk culture with added lactose

TIME OF DRYING	MOISTURE CONTENT OF CULTURE	SURVIVORS PER GRAM	
		Moist powder	Water free basis
hours	%		
0	59 05	785,000,000	1,917,000,000
0 5	48 05	750,000,000	1,443,000,000
1 0	34 71	963,000,000	1,475,000,000
1 5	24 05	942,000,000	1,240,000,000
2 0	10 56	916,000,000	1,024,000,000
2 5	4 74	351,000,000	368,000,000
	3 25	385,000,000	393,000,000

Data of Rogers, 1914

may contain as many as 700,000,000 viable bacteria per gram" (Fundamentals of Dairy Science, 1935, p 432) Commercial yeast cultures are usually dried on some cereal constituents, lactic cultures for dairy starters are sometimes dried on lactose, the rapid absorption of 5% water of crystallization by anhydrous lactose may be of help in rapid drying Bacteria in soil survive drying quite well Rahn (1907) found that 36% of the original flora of a good farm soil was still alive after 56 days of slow drying

The American Type Culture Collection uses drying quite extensively to preserve cultures, because it not only avoids the necessity of continual transfers, but also prevents the formation of variants, dissociants, mutants, etc Dried cells cannot possibly change their morphological or physiological characters

Different species exhibit quite different resistances to desiccation Stark and Herrington (1931) found that streptococci could tolerate the sudden change from moist to dry state (when the bacteria were dried in their culture medium) much better than *E coli* or *Lactobacillus acidophilus*, while yeast and staphylococci showed an intermediate tolerance

The survival of bacteria during the process of desiccation and afterwards is of importance in public health as well as in food preservation. In the manufacture of milk powder, for instance, not all bacteria are killed. Even the severe treatment of the drum-drying method, where the milk flows onto rotating, steam-heated drums and is scraped off as a paper-thin, dry sheet, leaves some vegetative forms alive. According to Supplee and Ashbaugh (1922), usually only one out of 10,000 bacteria survives. The very rapid spray-drying process yields a much higher survival ratio. The book of Hunziker (1935) has compiled a large number of data, and the plate counts of drum-dried powder range from 45 to 600,000 per gram while the spray-dried powders have the much higher limits of 4,400 and 5,500,000. A more recent survey of 671 English milk powder samples by Crossley and Johnson (1942) shows the wide extremes of 200 and 19,500,000 bacteria per gram.

Bacteria dried on metal surfaces die when in direct contact. The fact that many bacteria can be obtained from coins means only that there is a layer of protective dirt preventing immediate contact between bacteria and metal. Ordinary glass contains free alkali, and bacteria dried on coverglasses usually do not survive long.

Campbell-Renton (1941) tested the resistance of bacteriophage to desiccation and found that the decrease is approximately logarithmic. Different phages vary greatly in tolerance; of 15 phages for the dysentery bacteria, 8 were reduced to less than 1% of their original activity after 24 hours of drying, while 6 had more than 50% of their activity left. Once in a dry state, the viability decreases very slowly if the vacuum is maintained. Even after $3\frac{1}{2}$ years of storage over P_2O_5, some phages had lost little of the activity which was left after the initial decrease by the drying process as such. Most resistant were the phage "Pasteur" for *Staphylococcus aureus* and the phage "D M Large" for *Salmonella schottmuelleri*.

2. *Death of dry bacteria.* The death rate of dry bacteria was first studied by Th. Paul (1909) and by Paul, Birstein and Reuss (1910). *Staphylococcus aureus* was dried on garnets, and the cells died slowly, and in logarithmic order, when kept at room or incubator temperatures. The actual cause of death was found to be oxidation. Table 3 illustrates the effect of the oxygen concentration on the death rates which were computed from frequent plate counts. The bacteria of Series A were kept in air and in commercial oxygen while in Series B, an intermediate oxygen concentration was used. The concentration exponents[2] average 0.44 which means that the death rate is approximately proportional to the square root of the oxygen concentration. Rogers (1914) also found a higher death rate in air or in oxygen than *in vacuo*, in hydrogen or carbon dioxide. However, bacteria die also in the complete absence of oxygen, though quite slowly. The cause of this death has never been studied.

[2] The concentration exponent n is calculated from the ratio of two different concentrations, and from the ratio of the corresponding death rate constants, by the equation $\left(\frac{C_1}{C_2}\right)^n = \frac{K_1}{K_2}$

The order of death is essentially logarithmic as may be seen from fig 5a which shows the average decrease of viable bacteria in 9 samples of milk powder kept at 5 different moistures The break at about 500 bacteria per gram may

TABLE 3

Death rate constants of dried staphylococci at different temperatures and oxygen concentrations

SERIES	OXYGEN CONCENTRATION	DEATH RATE CONSTANTS = $\frac{1}{t}$ LOG $\frac{\text{INITIAL NUMBER}}{\text{SURVIVORS}}$ at 18.2 C	at 24.9 C	at 37 4 C	Q_{10}
	%				
A	20 8	0 0017	—	0 0157	3 3
	96 2	0 0034	—	0 0256	2 7
Concentration exponent n		0 46		0 32	
B	20 8	—	0 0107	0 0264	2 1
	54 6	—	0 0152	0 0369	2 0
	96 2	—	0 0200	0 0444	1 5
Concentration exponent n			0 36 0 50	0 56 0 46	

Data of Paul, Birstein and Reuss, 1910

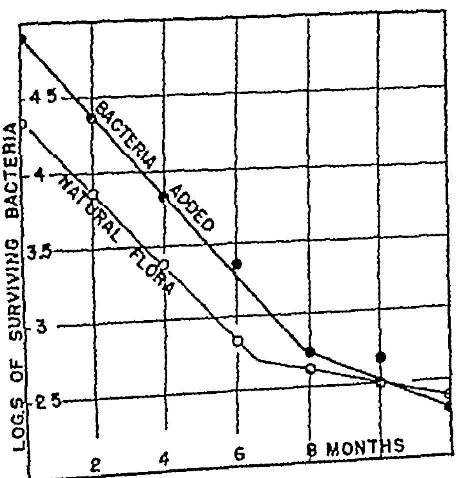

Fig 5a

Decrease of bacteria in milk powder (Data of Supplee and Ashbaugh, 1922)

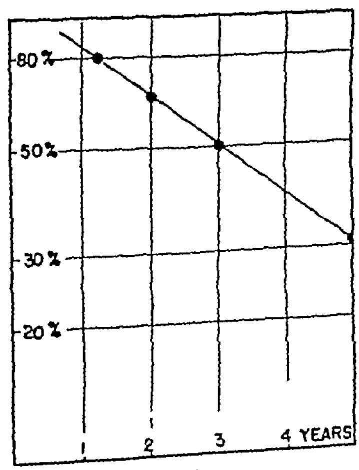

Fig 5b

Decrease in activity of dry smallpox vaccine *in vacuo* (Data of Otten, 1933)

be due to spores or very resistant species Fig 5b shows the decrease of viability of dried smallpox vaccine kept in high vacuum at room temperature in the tropics, according to Otten (1933)

The temperature coefficients of the death of dry bacteria are those to be expected for an oxidation process The data of table 3 give an average Q_{10} of

about 3 for series A, and of 2 for series B Rogers' (1914) experiments with dried lactic cultures show a Q_{10} of 3 6 between 0 and 30 C

The survival of pathogenic bacteria, e g from dried feces, or spray-dried by coughing and sneezing, must be known in order to prevent epidemics The bacteria remaining viable on dried vegetables and meats, on milk powder and egg powder decide how readily the food will spoil when moistened Sugar contains usually the very resistant spores of thermophilic bacteria which spoil canned vegetables because the spores survive the canning process While in some cases, we employ drying in order to kill as many bacteria as possible, we utilize, on the other hand, the longevity of dried bacteria as a great help in

TABLE 4

Survivors of dried cholera vibrios, per million cells

STRAIN OF VIBRIO	SURVIVORS AFTER		
	24 hours	5 months	4 years
Shillong 653	1,950	284	26
Shillong 1077	12,700	4,200	5 2
Shillong 610 R	4,630	2,600	0
Shillong 610 R	1,960	155	0
Rangoon R	14,200	450	12
Inaba S	1,950	—	97
Inaba R IV	16,000	—	7,000
Rangoon S	62	—	3 2
El Tor D 12	11,000	—	50
El Tor D 12	21	—	15
El Tor D 31	4,100	—	30
El Tor D 35	51,000	—	550
El Tor D 35	100	—	12
El Tor D 6	2,150	—	0
El Tor D 33	4,700	—	0

Data of Campbell-Renton, 1942

providing cultures for commercial purposes magic yeast, starters for dairy purposes, and nodule bacteria dried on soil

Bacteria are also dried to prevent them from changing their properties by mutation, dissociation or adaptation Most of these cultures, if they are to remain alive for a very long time, are kept in a vacuum and at very low temperatures How they gradually decrease, may be seen from Campbell-Renton's (1942a) experiences with the very sensitive cholera vibrios kept at room temperature in a high vacuum over P_2O_5 (table 4)

3 Death by dry heat Dry cells display no life functions, the enzymes are not active in the absence of moisture, even endogenous catabolism has ceased The cells die from oxidation, and when the temperature is raised above the maximal temperature of the species under test, death is still due only to oxidation There is no coagulation of proteins because dry proteins do not coagulate when heated to 100 C, and dry enzymes retain their activity All experiments show that at

higher temperatures, bacteria die more rapidly, but the gradual increase in the death rate is due merely to an increase in the rate of oxidation, there is no abrupt change in the death rate at the maximum temperature of growth, nor at any other point

Otten (1930) found dried typhoid, dysentery and cholera bacteria able to survive 37 C for many months, 42 C for several weeks, 58 to 60 C for 7 to 10 days, and even 100 C for 1 to 2 hours Boysen (see Rahn, 1932 p 309) measured the death rate of yeast dried on infusorial earth and on sand The temperature coefficient between 60 and 98 C fluctuated between 4 2 and 6 8 while between 30 and 50 C, it varied from 2 1 to 4 1

Though dry heat is used in all laboratories to sterilize Petri dishes, pipettes and other equipment, there seems to be a surprising lack of a systematic study

TABLE 5

Death times (in minutes) of the spores of Clostridium botulinum exposed to dry heat

TEMP	I	II	III	IV	V
C					
110°	>120	>120	>120	>140	115–120
115°	>120	>120	>120	>110	80– 85
120°	110–115	95–100	110–115	>120	100–105
125°	>60	>60		40–45	45– 50
130°	55– 60	30– 35		40–45	55– 60
135°	35– 40	35– 40		>65	>65
140°	30– 35	40– 45	60– 65	15–20	15– 20
145°	25– 30	15– 20	25– 30		10– 15
150°	20– 25	15– 20	25– 30		10– 15
155°	10– 15	10– 15	25– 30	5–10	10– 15
160°	20– 25	15– 20	20– 25	10–15	10– 15
165°			15– 20		
170°			10– 15		
175°			5– 10		
180°			5– 10		

Data of Tanner and Dack, 1922

of the death rates at high temperature In 1921, Ayers and Mudge measured the death times of *E coli* and of a heat-resistant lactic type by drying aqueous suspensions from agar slants on tin strips, and placing these in wide test tubes in an oil bath They found that in order to be killed in 30 minutes, *E coli* needed 60 C in milk, but 71–82 C in hot air, the lactic type needed 76 C in milk, but 110 C in hot air, a sporeformer needed more than 132 C in hot air

All other experiments by these authors were made upon milk cans that were still wet when placed in hot air, so that no precise line between moist and dry heat could be drawn In the experiments by Dahlberg and Marquardt (1932) the dairy utensils to be sterilized in dry heat were also placed into the heater while still wet

The most detailed data that could be found are those of Tanner and Dack (1922) who swabbed sterile test tubes with cultures of *Clostridium botulinum*,

dried them, and determined the death times at temperatures ranging from 110 to 180 C The results are given in table 5

The temperature coefficients of death by dry heat are very low From the data of table 5, the temperature coefficients for the entire range are 1 75, 1 85, 1 65, 1 59 and 1 83 This may seem contradictory to the Q_{10} of about 3 shown in table 3 However, those coefficients refer to temperatures between 18 and 37 C As will be explained in the section on Death by Moist Heat, temperature coefficients decrease slightly with increasing temperature A reaction with a Q_{10} of 3 at 20 to 30 C will display at 160 to 170 C a Q_{10} of only 1 7 (see table 10) Therefore, the results of Tanner and Dack (table 5) and those by Paul *et al* (table 3) are not in disagreement

The obvious consequence of these low temperature coefficients is that an increase of 10 degrees does not reduce the heating time greatly With a Q_{10} of 1 7, the temperature must be raised 13 degrees in order to halve the sterilizing time This low coefficient is the reason why the various laboratory manuals disagree widely on the times and temperatures necessary to sterilize dry glassware There is no disagreement about the sterilization of media in the autoclave because the temperature coefficient in this case is so high that an increase of 2 to 3 C cuts the sterilizing time in half

IV DEATH BY LOW TEMPERATURES

1 Subminimal temperatures Most bacteria cease to grow at temperatures 5 to 10 C above the freezing point, and when they cannot grow, they die without being frozen, though very slowly Hilliard and Davis (1918) suspended cells of *Escherichia coli* in glucose solutions and subjected them to temperatures as low as −6 C which did not freeze the solution About 50% of the cells died in 3 hours while in parallel suspensions in water which crystallized, 93 to 99% of the cells were killed *Streptococcus lactis* which cannot multiply at temperatures below 5 C was held by Rahn and Bigwood (1939) at 0 to 2 C The original number of 227 million cells per ml of milk decreased in 114 days to

16,000 per ml when the culture was not treated,
34,000 per ml when the culture was neutralized at the start,
450,000 per ml when the air was replaced by nitrogen,
63,000,000 per ml when the culture was neutralized and kept under nitrogen

Apparently, death is primarily due to a change of some essential cell constituent by oxidation which is prevented or repaired above the minimum temperature, i e as long as the temperature permits the synthetic mechanisms of the cell to function At 0 C, the oxygen concentration is twice as high as at 30 C

2 Cold shock Bacteria may also die from cold shock Sherman and Cameron (1934) could kill about 95% of very young cells of *E coli* by cooling them very suddenly from 45 C to 10 C while gradual cooling during 30 minutes caused no injury Some other species were less sensitive In older cultures, only a small percentage of the cells died The cause of death is not known Bělehrádek (1935 p 147) states "Under the action of cold, the cellular content is some-

times displaced in an atypical way" Several examples are given It seems probable that the suddenness of chilling is likely to enhance such displacement

Two experiments on the effect of cold shock upon higher organisms are available for comparison Kylin (1917) observed complete cessation of plasma streaming in the alga *Nitella clavata* after sudden cooling from 20 to 3 C Here as with bacteria, the emphasis is on suddenness It is imaginable that a very rapid temperature change disrupts the cell mechanisms either by upsetting chemical equilibria, or by spatially disconnecting some cell functions which depend upon each other The observation by Plough (1942) that temperature shocks increase the mutation rate, seems less likely to explain the death of such a high percentage of bacteria although we have learned to look upon death of bacteria as a lethal mutation (see p 9)

TABLE 6

Death by continuous freezing and by alternate freezing and thawing

(Numbers indicate plate counts per ml)

CONTINUOUS FREEZING		ALTERNATE FREEZING	
	Eberthella typhosa		
Inoculum	40,896	Inoculum	40,896
24 hrs	29,780	Frozen 3 times	90
3 days	1,800	Frozen 5 times	0
4 days	950	Frozen 6 times	0
5 days	2,490		
	Serratia marcescens		
Inoculum	339,516	Inoculum	339,516
24 hrs	36,410	Refrozen once	2,570
30 hrs	41,580	Refrozen 2 times	275
48 hrs	14,440	Refrozen 3 times	15
96 hrs	4,850	Refrozen 4 times	0

Data of Hilliard and Davis, 1918

3 Freezing When the water surrounding the bacteria changes to ice, the water inside of the cell usually solidifies too, as its freezing point does not differ greatly from that of the medium Solidification of the water prevents any kind of metabolic action, and there is some analogy between frozen bacteria and dry bacteria In both cases, the act of transferring bacteria from the normal into the anhydrous state is a severe ordeal and kills many cells, but those which survive die at a very slow rate if kept anhydrous Hilliard and Davis (see table 6) compared alternate freezing and thawing with the holding of frozen bacteria at −1 C After freezing and thawing 5 successive times, all cells in cultures of *E typhosa* and *Serratia marcescens* were dead while after remaining undisturbed in the frozen state for 4 days, several thousand cells remained alive, and most of those that were dead had been killed during the initial freezing

The greatest injury by the act of freezing must be due to the change of water to ice which is accompanied by expansion, by crystal formation, and by colloidal changes Expansion is not so likely to injure the rather elastic cell, but sharp-edged ice crystals may puncture the plasma membrane Some colloidal solutions, after freezing and thawing, remain unchanged while others show a separation of the concentrated colloid from the liquid phase, the melted crystals Such separation is commonly observed in frozen cells of plants and animals, and results in death of the cell

Crystallization requires the presence of seed crystals or the formation of crystallization nuclei by a special collision of water molecules The probability of such collisions is greatly reduced by colloids According to Callow (1925), the addition of 3% gelatin to water reduces its velocity of crystallization to 1/350 of the normal rate Ice formation in cells will therefore be slow The number of nuclei per cell will also depend upon the volume involved, and ice formation in bacteria will be slower than in the much larger cells of plant leaves This may account for the fact that as a rule, not all cells in a suspension of bacteria are killed by a single freezing

Freezing involves several causes of death, and the most common cause, injury by ice crystals, is quantitatively unpredictable Thus no order of death can be expected, and no order has been observed The survivors of the freezing process die at a very slow rate when kept in the frozen state Bacteria, yeast cells and mold spores have been known to survive for several years in the frozen condition It is impossible to sterilize foods or even water by freezing

A different picture is obtained when freezing is accomplished very rapidly to very low temperatures, e g by immersion in liquid air Then, the water in the cells is not changed to ice crystals, but to a glass-like, amorphous mass Luyet and Gehenio (1940) speak of this change as vitrification, and of the physical state as vitreous Water in the vitreous state may change to the crystalline ice stage, and this happens more readily at higher temperatures, when the vitreous mass is warmed slowly to a temperature near the freezing point If cells are successfully vitrified without formation of ice, they can be held at low temperatures for a very long time without danger of ice formation This danger exists, however, during the thawing process Bacteria which have survived vitrification, may thus be killed during the thawing Rapid thawing will prevent this

It is not surprising, therefore, that Kyes and Potter (1939) found tubercle bacteria to survive rapid freezing and thawing 20, 40, 80 and even 200 times when frozen in steel test tubes in liquid air, and thawed in hot water Only one experiment was made with CO_2-ice in glass tubes, and no growth *in vitro* could be obtained after 25 alternations of slow freezing and thawing Storage in the refrigerator for 6 years at -3 C killed all bacteria because the refrigerator was defrosted twice each year which meant twelve very slow freezings and thawings What seemed offhand to be a most severe treatment, namely, the rapid change over 200 C in liquid air, proved to be rather harmless, thanks to vitrification The literature on this point, and on freezing generally, has been reviewed critically by Luyet and Gehenio (1940)

V DEATH BY MOIST HEAT

Heat is applied in two different ways for the destruction of bacteria Glassware and certain instruments and materials are sterilized with dry heat The effect of high temperatures on dry bacteria has been discussed in the preceding pages Other materials are heated when wet, e g , foods in the canning process, milk, beer and wine during pasteurization, culture media for bacteria, and surgical dressings For this process, the term "moist heat" is most commonly used although Chick, in the first detailed quantitative study, spoke of it as "death by hot water" The cause of death in moist heating is quite different from that in dry heating, and the rules applying to the one method do not fit the other Death by dry heat is primarily an oxidation process, death by moist heat is due to coagulation of some protein in the cell

1 Thermal death point and thermal death time The standard of comparison of heat tolerance of different species was originally the Thermal Death Point, i e , the lowest temperature at which a suspension of bacteria is killed in 10 minutes (see Descriptive Chart, Society of American Bacteriologists) This *method cannot* give comparable results unless the conditions are standardized as to age of culture, approximate number of cells, pH of suspension, dimensions of test tubes and thickness of glass in the test tubes

Research workers in the canning industry found it more suitable for their purposes to keep the temperature constant and to vary the time Thermal Death Time is the shortest time necessary to kill all bacteria in a given suspension at a given temperature Bigelow and Esty (1920) suspended the bacteria or spores to be tested in clear juices from canned foods, distributed the suspension uniformly among a number of small narrow glass tubes, sealed these completely by fusing the glass, and dropped them into an oil bath of constant temperature Tubes were removed at different times and incubated, survival of any bacteria became evident by clouding of the medium A review of various slight alterations of technique is given by Beamer and Tanner (1939a) It is necessary to determine the initial number of cells or spores, because the thermal death time is longer with larger inocula

2 The order of death The order of death by heat is logarithmic From the earliest quantitative measurements by Chick (1910) to the extensive investigations by Watkins and Winslow (1932), death of vegetative cells as well as death of spores has been found to be logarithmic The investigations by Bigelow and others of the National Canners Association and the many studies of heat sterilization of spores of *Clostridium botulinum*, e g , by Weiss (1921) and Esty and Meyer (1922) have confirmed this A number of experiments by Beamer and Tanner (1939a, b) with vegetative cells of bacteria and with yeasts gave the same order

The customary explanation that death is brought about by heat inactivation of the enzymes (see e g , Isaacs, 1935) cannot be correct because, for mathematical reasons, a logarithmic order is possible only when death is due to the destruction of a single molecule in the cell (Rahn 1929, 1943) To be sure, Edwards and Rettger (1937) found that washed cells of bacteria, held for 24

hours between 40 and 60 C, lost all their respiratory enzymes at temperatures near the maximum for growth However, enzyme deterioration of washed cells without food held for such a long time under very abnormal conditions permits no conclusions as to the behavior of the same cells in a suitable medium Rahn and Schroeder (1941) repeated the experiment using the method of Edwards and Rettger for enzyme analysis, but they measured viable cells and enzyme content *in the same sample* of cell suspensions of *Bacillus cereus* suspended in phosphate buffer at 46 C and 50 C The first line of data in table 7 shows that 99% of the cells were dead when only 14% of the peroxidase and 20% of the catalase had been inactivated Enzyme coagulation could not possibly have been the cause of death Similar results had been obtained with yeast by Rahn and Barnes (1933)

TABLE 7

Death of cells and loss of enzyme activity of Bacillus cereus under the action of heat

TEMPERATURE	TIME OF EXPOSURE	VIABLE CELLS (PLATE COUNT)	CATALASE	DEHYDROGENASE
Percentage of cells or enzymes remaining				
C	*minutes*			
46	10	1 1	80	86
	20	0 04	65	86
	40	0 002	48	67
	80	0 00001	38	46
50	5	2 4	—	89
	10	0 006	56	57
	20	<0 00002	56	9
	40	<0 000002	48	—

Data of Rahn and Schroeder, 1941

Microbial enzymes continue to function for a considerable time at temperatures above the maximum for growth Table 8 shows that centrifuged cells of *Streptococcus lactis* suspended in buffer solution with glucose ferment rapidly at 42 C and even at higher temperatures This strain multiplies most rapidly at 33 C and cannot multiply above 41 C, yet at 42 C, the energy available from fermentation is far higher than at 33 C Multiplication at 42 C cannot be handicapped by the heat inactivation of the enzymes, but by the inactivation of the synthetic catalysts or the cell division mechanism Similar data have been obtained with yeast which grows most rapidly at 29 C, ceases to grow at 35 C, but ferments at 44 C more rapidly than at 29 C (Rahn, 1932, p 132)

The mathematical necessity that death must be brought about by destruction of a single molecule brings us back to the definition given in the chapter on radiation that death is a lethal mutation We may assume that heat coagulation of a single gene prevents reproduction Such a cell is sterile, and according to bacteriological standards, it is dead, though its enzyme content may not be exhausted However, the inactivation of the growth mechanism is likely to make

repair and replacement of inactivated molecules impossible, and the enzyme content of such sterile cells must gradually decrease and this is evident in table 8 The decreasing enzyme content is the *consequence* of inhibited growth, and not its *cause*

3 Death rates Regardless of whether we accept the one or the other explanation of the cause of the logarithmic order of death, its existence permits us to compute *death rates* and to draw conclusions from them which are independent of any explanation Death rates make it possible to compare the heat resistance of different species at the same temperature, or the heat resistance of one species at different temperatures It also enables us to describe in quantitative terms the effect of environmental factors, such as concentration of the medium or its pH, upon heat sterilization

TABLE 8

Per cent lactic acid produced in buffer plus glucose by centrifuged cells of Streptococcus lactis (1,315,000,000 cells per ml)

TEMPERATURE	MINUTES										
	10	20	30	40	50	60	90	120	150	180	240
C											
30		0 036		0 072		0 108	0 144	0 189	0 234	0 279	0 360
33		0 059		0 107		0 144	0 198	0 252	0 306	0 360	0 437
37		0 072		0 135		0 189	0 261	0 315	0 369	0 405	0 481
40		0 108		0 180		0 243	0 315	0 385	0 441	0 486	0 549
42		0 099		0 171		0 243	0 315	0 385	0 437	0 473	0 495
45	0 054	0 099	0 144		0 207	0 221	0 297		0 351		0 369
47	0 063	0 099	0 130		0 198	0 216	0 270		0 306		0 306
50	0 036	0 063	0 090		0 108	0 117	0 144		0 171		0 171

Data of Dorn and Rahn, 1939

For the application of heat in the preservation of foods, the death rate constant is too cumbersome, and simpler constants have been introduced Baker and McClung (1939) measured the time required, at a certain temperature, to reduce the bacteria to 0 01% of their original number More elastic in its applications is the Decimal Reduction Time (D R T) by Katzin, Sandholzer and Strong (1942) which is the time required to reduce the bacterial population to 10% of the original number Accordingly, a doubling of D R T must reduce the population to 1% and heating for 4× (D.R T) reduces it to 0 01%, so that the constant of Baker and McClung is identical with 4 times the Decimal Reduction Time

Ayers and Johnson (1914), in their study of pasteurization of milk, observed that in cultures of *E coli* and of lactic streptococci, a very few cells were sometimes found which were much more resistant than the great average They introduced the term "majority thermal deathpoint" for the lowest temperature which kills the large majority of cells in 10 minutes This phenomenon had been described in detail by Gage and Stoughton (1906) who worked with *E coli*

Beamer and Tanner (1939a, b) gave a clearer picture by drawing the logarithmic survivor curves which showed a sharp break, indicating that the remaining cells had a much greater, but uniform resistance These experiments included, besides the colon-typhoid group, also several yeasts Gage and Stoughton could find no evidence of spore formation, and they demonstrated that the greater heat tolerance was not inheritable In all reported cases, the percentage of resistant cells has been less than 0 1 Whatever the explanation, the great practical importance of these very few highly resistant cells in commercial pasteurization of all kinds of foods is obvious Chambers and Gaines (p 8) had a similar experience when killing bacteria by sonic waves

Very complicated is the mathematical treatment used by Ball (1923, 1928) in the study of the temperatures required in the canning of vegetables This problem involves not only the heat sterilization of spores, but also the heat conductivity of the cans and their contents The z and F factors which play an important role in these equations will be discussed later

4 The temperature coefficient of death by moist heat The death rate constants are either obtained from plate counts at certain exposure times, from the equation $Kt = \log \frac{\text{initial number}}{\text{survivors}}$ or they may be computed from the thermal death times In this latter method, the initial inoculum for all tubes is the same for any series of experiments and the final number of survivors is also the same, namely less than 1, so that the quotient of initial number over survivors is constant [3]

If the death time is determined at the temperatures T_1 and T_2 (T_2 being higher), we may call the corresponding death rate constants K_1 and K_2, and the death times t_1 and t_2 Since the order of death is logarithmic, we have the formula

$$K_1 t_1 = \log \frac{\text{initial number}}{\text{survivors}} = K_2 t_2$$

or

$$\frac{K_2}{K_1} = \frac{t_1}{t_2}$$

This quotient indicates how much more rapidly death proceeds at the higher temperature T_2 This is the temperature coefficient for the temperature increase $T_2 - T_1$ For comparative purposes, it is customary to give the coeffi-

[3] The thermal death times are not precise values Between the last sample that showed viable bacteria and the first that showed none, some time has passed During this interval, the number of survivors was reduced to less than 1 per sample In all experiments, the number of survivors was identically the same at some moment between these two critical times, but the exact moment is not known All death time data have a certain range of possible error, the magnitude of which depends upon the spacing of the time intervals The number of survivors is never zero, but becomes very small, e g , 1 in 100 liters, 1 in 1,000 liters, etc

cient for an increase of 10 C which is designated as Q_{10} The formula for conversion from Q_n to Q_{10} is indicated in the following relation

$$Q_{10} = Q_n^{\frac{10}{n}} = \left(\frac{t_1}{t_2}\right)^{\frac{10}{n}}$$

A number of such temperature coefficients of heat disinfection have been compiled by Rahn (1932, pp 320–323) For spores, they are fairly uniform, between 8 and 10 at temperatures from 100 to 135 C With vegetative cells

TABLE 9

Temperature coefficients of disinfection by moist heat

	Q_{10} in nutrient broth, pH 7.05		Q_{10} in tomato juice, pH 4.2	
	Temperature interval			
	55–60 C	60–65 C	55–60 C	60–65 C
Eberthella typhosa	28 9	26 1	20 2	2 7
Salmonella paratyphosa	37 7	10 3	—	—
Salmonella scottmuelleri	42 0	33 7	3 3	—
Salmonella aertrycke	23 7	9 0	5 2	27 7
Salmonella enteritidis	36 0	15 3	9 8	—
Staphylococcus aureus	28 9	59 2	10 1	3 9

	Q_{10} in broth, pH 6.8		Q_{10} in broth, pH 3.8		Q_{10} in grape juice, pH 3 6	
	Temperature interval					
	55–60 C	60–65 C	55–60 C	60–65 C	55–60 C	60–65 C
Debaryomyces globosus	4 0	—	12 2	—	6 5	—
Monilia candida	4 0	—	71 0	—	63 0	—
Saccharomyces ellipsoideus	11 6	12 2	15 4	9 0	21 3	19 6
Torula monosa	20 6	7 0	7 5	8 0	6 4	29 2

Data of Beamer and Tanner, 1939a, b

at 50 to 80 C, they are usually higher Some more recent data for vegetative cells are given in table 9

The coefficients computed from the data by Beamer and Tanner fluctuate greatly, each value being the result of only one experiment This fluctuation is due to the fact that the determination of death rates generally is subject to considerable experimental error, the possibility of error is greatly enlarged when the quotient of two such death rates is computed Most of the coefficients are above 10, and where they are lower, it is due to the survival of a few individuals with much higher resistance, e g , in the case of *Debaryomyces* and *Monilia* (Rahn, 1943) The high temperature coefficient makes it practically certain that death by heat is a coagulation (or denaturation) process, as such high temperature coefficients are very rare except with protein coagulation

Temperature coefficients are not really constant The thermodynamic definition of the temperature coefficient is

$$Q_{10} = \frac{\text{RATE at } T + 10^\circ}{\text{RATE at } T^\circ} = \frac{K_{T+10}}{K_T} = e^{\frac{\mu}{2}\frac{10}{T(T+10)}}$$

where e is the base of the natural logarithms and μ is the temperature characteristic of the reaction It is this value μ that is constant As T, calculated

TABLE 10

The temperature coefficient decreases with rising temperature while the temperature characteristic μ remains constant

above "normal" chemical reactions

below denaturation of proteins

TEMP RANGE	$\mu = 12{,}286$	$\mu = 19{,}506$	$\mu = 24{,}610$
C			
betw 0– 10	2 22	3 49	4 92
10– 20	2 10	3 20	4 42
20– 30	2 00	3 00	4 00
30– 40	1 91	2 76	3 66
40– 50	1 84	2 60	3 38
100–110	1 53	1 98	2 37
150–160	1 40	1 70	1 96
160–170	1 38	1 66	1 90
170–180	1 36	1 62	1 84
	$\mu = 49\ 600$	$\mu = 64\ 600$	$\mu = 99{,}200$
betw 50– 60	10 0	20 0	100 0
60– 70	8 8	17 0	76 7
70– 80	7 8	14 5	59 6
80– 90	7 0	12 4	47 9
90–100	6 3	10 9	38 9
100–110	5 6	9 6	32 0
110–120	5 2	8 6	27 0
120–130	4 8	7 7	23 0

as absolute temperature, increases, the value T (T + 10) becomes larger, and therefore the exponent of e becomes smaller, consequently, Q_{10} decreases with increasing temperature

Table 10 shows how the temperature coefficient of the same reaction changes when the temperature varies The upper part of the table shows that reactions with a Q_{10} of 2, 3 and 4 at 20 to 30 C will have at 170–180 C the much smaller coefficients of 1 36, 1 62 and 1 84 In sterilization by dry heat, where oxidation is the cause of death, a "normal" temperature coefficient of about 3 can be expected, but this value 3 refers to room temperature, and an increase from 170 to 180 C will increase the rate of death only 1 6 times

The lower part of table 10 deals with protein reactions such as coagulation, denaturation, or inactivation, which have very high temperature coefficients. A coefficient of 100 at 50 C corresponds to a Q_{10} of 23 at 120 C. The fact that the Q_{10} of spore sterilization in canning averages about 9, while that of milk pasteurization is about 20, does not prove that we are dealing with different reactions. The same reaction with a Q_{10} of 20 near 60 C has at 120 C a Q_{10} of 8.

So long as temperature effects are studied within the narrow limits of life, i.e., between 10 and 50 C, the assumption of a constant Q_{10} does not introduce a great error, but when the range becomes as wide as in the study of death by heat, the assumption of a constant temperature coefficient may be very misleading.

Two different methods of pasteurization of milk are permitted by the New York State Department of Health, namely the holding process which consists of holding the milk for 30 minutes at 143 F, and the short-time, high temperature process, usually called the flash heat process, which requires holding for 15 seconds at 160 F. The temperatures are 9.44 C apart (71.11–61.67) and the heating time of the one process is 120 times that of the other. This means, that the authorities assumed a $Q_{9.44}$ (which is practically Q_{10}) of 120, a very high temperature coefficient for this range.

It is obvious that the temperature coefficients of death cannot be extrapolated into the region of growth. If a bacterium can multiply, though very poorly, at 40 C, but dies at 50 C, the temperature coefficient will equal *the death rate* at 50 C divided by zero which means $Q_{10} = \infty$. A sharp drop from infinity to the actual temperature coefficient of inactivation of the life processes must be expected, and very high coefficients must be expected at the lowest lethal temperatures. This accounts for the high coefficients found for *Escherichia coli* near 50 C since its maximum temperature of growth is 47 C. Watkins (1933) computed the increase of death rate between 50 and 55 C to correspond to a Q_{10} of 560. Baker and McClung (1939) found for the same bacterium in three experiments:

between 51.7 and 54.5	Q_{10} = 290.0	315.0	16.6
between 54.5 and 57.3 C	22.7	21.6	408.0
Average between 51.7 and 57.3 C	86.7	70.8	89.2

Henderson Smith (1923) computed from his experiments with the spores of *Botrytis cinerea* the following temperature coefficients:

between 31 and 37 C	Q_{10} = 690
between 37 and 44.3 C	132
between 44.3 and 47.0 C	92.8
between 47.0 and 50.3 C	29.5

The data on the death rates of spores show a *far greater* uniformity and constancy than those for vegetative cells, because all data on spores refer to temperatures which were at least 30 C higher than the maximal growth temperature,

while many of the experiments with vegetative cells were made within 10 or 15 C of the maximum for growth

5 Factors controlling death by heat Sensitivity to heat varies with the species Pasteurization of milk by holding at 63 C for 30 minutes destroys all pathogenic bacteria except the spores, and kills many species of saprophytes, but not all With some species such as lactobacilli, the death rate is low, and sometimes, thermophilic bacteria multiply during pasteurization The spores of many species can survive a short period of boiling, but the spores of *Methanobacterium omelianskii* are killed at 80 C in 10 minutes (Barker 1940) The cause of the great resistance of bacterial spores will be discussed at the end of this chapter

The medium in which bacteria are suspended can influence the death rate The best-known example is the effect of the acidity of the medium Bacteria die more rapidly in acid or alkaline media than in neutral suspensions This suggests, of course, the assumption of chemical disinfection rather than physical coagulation, but two facts speak against this In chemical disinfection, the temperature coefficients should be relatively low while table 9 shows that they are, on the average, higher in acid than in neutral media Further, yeast which grows better in acid media is killed by heat more easily in acid than in neutral media (Beamer and Tanner, 1939b)

Extensive experiments on the effect of pH on the heat resistance of spores have been carried out by Weiss (1921), Esty and Meyer (1922) and others The study by Townsend, Esty and Baselt (1938) may serve as a more recent example These authors did not use death rates or temperature coefficients, but the factors F and z which Ball had introduced in 1923 for the computation of processing times in the canning industry As these two values are frequently used in the canning industry which furnishes the largest amount of data for death by heat, the meaning of these factors will be discussed here

The value F is the thermal death time of the species at 121 C or 250 F The number z is the temperature increase, in degrees Fahrenheit, necessary to reduce the death time to one-tenth We introduce the temperature coefficient for 1 C, Q_1, which can be transformed into Q_{10} by the simple relation $Q_{10} = Q_1^{10}$ A few pages earlier, it has been shown that the ratio of the death rates at temperatures T_2 and T_1 can be measured by the corresponding death times, viz ,

$$\frac{K_2}{K_1} = \frac{t_1}{t_2}$$

This ratio is the temperature coefficient for $n° = T_2 - T_1$ degrees, hence

$$Q_n = Q_1^n = \frac{t_1}{t_2}$$

The definition of z specifies that with an increase of z°F, or 0 555z°C, the death time t_2 is one-tenth of t_1 Therefore, $n = 0\ 555z$, and $\frac{t_1}{t_2} = 10$

$$Q_1^n = Q_1^{0\ 555z} = \frac{t_1}{t_2} = 10$$

$$0\ 555z \log Q_1 = \log 10 = 1$$

We have seen that $Q_{10} = Q_1^{10}$, therefore

$$\log Q_{10} = 10 \log Q_1 = \frac{10}{0.555z} = \frac{18}{z}$$

$$z = \frac{18}{\log Q_{10}}$$

The value of z or Q_{10} indicates the slope of the straight line obtained by plotting the logarithms of death times against temperature The value F gives one point on this curve, and thus, F and z (or its corresponding Q_{10}) are sufficient to characterize the thermal resistance of the spores of a species at any temperature

The authors studied in detail the heat resistance of the spores of two strains of *Clostridium botulinum*, and of a bacillus isolated from spoiled canned corn The summarized results are given in table 11 where the z values have been converted into Q_{10} With the bacillus spores, the value F, the death time at 121 C (250 F), was not greatly affected by the medium, but the spores of the two clostridia survived in the neutral phosphate buffer 2 to 4 times as long as in milk and in the vegetable juices The differences cannot be explained by pH alone It has been observed in commercial canning that the heat tolerance of spores is not the same in different vegetables, but the cause is not known The temperature coefficient with the spores of the two clostridia was higher in the vegetable juices than in the buffer, whereas the spores of the bacillus showed the opposite In the buffer, the three behaved nearly alike Tanner (1944, p 962) speaks of a "food factor" which is the ratio of death time in food juice to death time in neutral phosphate buffer

TABLE 11

The heat resistance of the spores of three bacteria, expressed by the values F and Q_{10}

MEDIUM OF SPORE SUSPENSION	pH	BACILLUS 3679		C. BOTULINUM 62A		C. BOTULINUM 213B	
		F*	Q_{10}	F*	Q_{10}	F*	Q_{10}
Phosphate buffer	7 0	4 00	12 1	1 70	12 5	2 00	10 0
Asparagus	5 4	3 30	7 1	0 39	15 9	0 39	15 9
Peas	5 4	3 00	11 6	0 30	22 0	1 40	14 2
Spinach	5 4	2 60	9 8	0 65	14 5	0 68	14 5
Milk	6 3	2 60	7 8	0 45	16 8	0 50	18 2
Average			9 7		16 3		14 6

* Death time in minutes at 121 C

Experiments of Townsend, Esty and Baselt, 1938

Different from this chemical effect is the protection afforded by concentrated solutions The well-known increase of the thermal resistance of bacteria through addition of sugar is usually explained by partial dehydration of the protoplasm In concentrated solutions of sugar, proteins are not coagulated by heat (Beilinsson, 1929) According to Fay (1934), egg albumin which coagulated in 3 to 4 minutes at 60 C, required 23 and 31 minutes respectively in molar solutions

of glucose and sucrose, and 73 minutes and more than 24 hours, respectively, in twice molar solutions Rennet enzyme dissolved in water was completely inactivated at 70 C in 15 minutes, but molar $CaCl_2$ protected it for 45 minutes, and in 4 molar sucrose it lost only half of its strength in 5 hours K von Angerer and Küster (1939) confirmed the assumption that bacteria become partly dehydrated by comparing the turbidities of bacterial suspensions in water and in concentrated solutions

The time of contact is of importance Fay obtained only 2 5% survivors of *Escherichia coli* upon heating immediately after suspending the cells in 50% sucrose, whereas the same heating left 79% of the cells unharmed if they had been in the sugar solution for 2 hours before heating

TABLE 12

The harmful effect of high temperature on liquids containing reducing sugars (in half-molar concentration)

Survivors of *E coli* after being heated for 8 minutes at 54 C in variously treated sugar solutions at pH 7 0

CONCENTRATION	SUGAR	PERCENTAGE SURVIVORS IN MEDIUM PREVIOUSLY STERILIZED BY			
		Autoclaving		Filtration	
		In buffer	In broth	In buffer	In broth
%					
0	(control)	1 3	7 2	1 9	3 9
9	glucose	0 08	2 7	5 3	7 9
9	galactose	0 04	1 8	4 3	8 0
17 3	lactose	0 09	1 6	18 0	11 8
17 3	maltose	0	3 1	5 0	7 3
17 3	sucrose	9 2	15 0	12 0	10 0
9 1	mannitol	4 5	10 8	5 5	8 9
4 1	glycerol	3 2	9 4	3 7	6 4

Data of Baumgartner, 1938

Some inconsistencies in the results by various investigators are explained by Baumgartner (1938) as being caused by harmful decomposition products originating from reducing sugars during autoclaving This is prevented by aseptic filtration Table 12 shows that the percentages of survivors in the last three solutions, representing non-reducing compounds, are nearly the same in autoclaved and filtered media while the four media containing reducing sugars have become quite toxic by autoclaving

The theory of dehydration of the protoplasm is too simple to fit the facts Protection is not proportional to molarity (table 12), disaccharides protect more than monosaccharides but maltose seems to be an exception Von Angerer and Küster (1939) measured the death rates of *Escherichia coli* and *Staphylococcus sp* at 56 C in concentrated solutions of various carbohydrates and other easily soluble substances It was found that starch, gum arabic, urea, pyruvate,

glycocoll, calcium chloride, or phosphate buffer offered no protection while glycol, glycerol, sorbitol and various mono- and di-saccharides retarded death very noticeably.

The Unusual Resistance of Spores is not limited to heat. It is very conspicuous with disinfectants, but does not extend to irradiation (see p. 13). It has been the cause of many investigations because bacterial spores are the most resistant organisms existing. The spores of molds, although more hardy than the mycelium, are killed readily by boiling, and the spores of yeasts are only slightly more resistant than the vegetative cells.

The oldest explanation, the assumption of a practically impermeable spore wall, can hardly be applied in the case of heat. A wall of only 0.1 μ thickness could not possibly insulate the cell contents against high temperature for several hours. Nor could such extreme lack of heat conduction protect spores e.g., for 440 minutes at 100 C, but for only 5 minutes at 120 C. Absence of moisture has been another explanation. According to early investigations, the specific gravity of spores seemed to be about 1.35 to 1.4 which indicated a low moisture content. Since dry proteins are not coagulated by heat, this explanation seemed plausible. However, newer measurements indicate that spores contain nearly as much moisture as vegetative cells (Virtanen and Pulkki, 1933, Henry and Friedman, 1937). K. von Angerer and Küster (1939) could extract water from spores by suspending them in concentrated sugar solutions. Virtanen and Pulkki assumed that the enzymes of the spores were in an inactive form, and therefore resistant. Friedman and Henry (1938) explained this resistance as due to the large amount of bound water in the spores. From measurements of the freezing points of spore suspensions in water and in sucrose solution, the bound water for three different species of bacilli could be calculated to 69, 63 and 59% of the weight of moist spores whereas the corresponding figures for vegetative cells were 0, 18 and 28%. The calculations are based on temperature differences of a few hundredths of one degree, and probably have a large experimental error.

O. B. Williams (1929) approached the problem from a different angle. He showed that the ash content of spores is low, and as proteins do not coagulate readily in the absence of electrolytes, the low electrolyte content of the spores may be responsible for their heat resistance.

A different viewpoint was investigated by Rosel von Angerer (1939) who considered the fairly high fat content of spores as a possible means of protection. Long extraction with fat solvents decreased heat resistance while impregnation with paraffin (dissolved in carbon tetrachloride) doubled it. Presence or absence of oxygen did not affect the death time. She could not verify the theory of Sobernheim and Mündel (1938) that spores adsorbed on porous materials such as soil are much more resistant. Of all the materials tested, only granulated coal increased the death time significantly.

However, the most probable explanation is the assumption that the protein in spores is different from that of vegetative cells (Heim, 1938). The temperature of denaturation of different proteins is by no means the same. Among

enzymes, cytochrome oxidase is inactivated at 53 C, trypsin at 66 C, urease between 70 and 80 C, while ribonuclease and taka-diastase can tolerate a short period of boiling It seems possible that during sporulation, the protoplasm of the cell is so altered that it is not easily denatured, during spore germination, this process would be reversed It is imaginable that this change is somehow connected with changes in bound water

TABLE 13

Relative resistances of bacterial and mold spores, and of viruses, referred to the resistance of E coli as unity

STERILIZING AGENT	ESCHERICHIA COLI	BACTERIAL SPORES	MOLD SPORES	VIRUSES AND BACTERIOPHAGE
Phenol	1	100,000,000[a]	1– 2[f]	30[b]
Formaldehyde	1	250[b]		2[i]
Dry heat	1	1,000[c]	2– 10[g]	±1[j]
Moist heat	1	3,000,000[d]	2– 10[g]	1– 5[k]
Ultraviolet	1	2–5[e]	5–100[e]	5–10[e]

[a] Chick (1908) gives death rate k = 0 0466 for anthrax spores with 5% phenol, and k = 0 212 for paratyphoid bacteria with 0 6% phenol Assuming concentration exponent n = 6, the ratio is $\left(\frac{5}{0\ 6}\right)^6 \frac{0\ 212}{0\ 0466} = 10^8$ 1

[b] Formaldehyde, 5%, kills anthrax spores in 32 hours while *E coli* is killed by 2% in 20 minutes (Chick, 1908) Assuming concentration exponent n = 1, the ratio is 250 1

[c] No records could be found on death rates of dried *E coli* and of dry bacterial spores at the same temperature If we assume that dry spores at 160° die at about the same rate as dry *E coli* at 60°, the death rate of *E coli* at 160° (100° increase, Q_{10} averaging 2) would be 2^{10} ≅1000 times as high as that of spores

[d] No records could be found on death rates of *E coli* and of bacterial spores in water at the same temperature If we assume that moist spores at 120° die about as rapidly as *E coli* at 60°, the death rate of *E coli* at 120° would be obtained by multiplying the rate at 60° with the average temperature coefficient raised to the 6th power From Table 10, the average Q_{10}, with μ = 64,000, is about 12 The death rate of *E coli* is therefore about $12^6 \cong$ 3,000,000 times as great as that of spores

[e] See p 13

[f] Woodward *et al* , 1934

[g] Own experience

[h] From data in McCulloch, p 353

[i] From data in McCulloch, p 373

[j] See p 19

[k] From data in McCulloch, p 118–119

Table 13 shows how many times more resistant bacterial spores, mold spores, viruses and bacteriophages are than *E coli* A comparison of this kind can give only the order of magnitude, because the spores of different bacteria are not equally resistant, the spores of different molds differ widely in resistance to ultraviolet, and the phages and viruses do not react identically either But the great contrast between bacterial spores and all the other forms is quite striking, and the absence of this contrast with ultraviolet and the differences within each group are suggestive of the different lethal reactions causing death

Experimentation with the resistance of spores is complicated by a great variability of this property due to environmental conditions A summary of the literature is given by Theophilus and Hammer (1938) Bacterial spores will occasionally remain dormant for a long time before germinating This accounts for the difficulty encountered in the sterilization of food by intermittent heating on successive days An extensive study has been made by Morrison and Rettger (1930) According to Evans and Curran (1943) incubation of spores at sublethal temperatures, e g , 65 C, accelerates the rate of their germination

VI SURFACE TENSION DEPRESSION

1 Effects of surface tension depression Many compounds which lower the surface tension of water are good disinfectants Best known among these are

TABLE 14

Surface tension of solutions killing Staphylococcus aureus in 5 minutes

	CONC.	SURFACE TENSION IN DYNES	
		In water	During test
	%		
Phenol	1 25	41 5	42 0
o Cresol	1 00	37 5	37 5
Hexyl resorcinol	0 025	37 5	37 5
Thymol	0 0625	38 5	38 5
Chlorothymol	0 25	49 0	49 0
Mercuric chloride	0 025	70 0	68 5
Merthiolate	0 025	51 0	51 5
Mercurochrome	1	71 5	65 0
Iodine	0 02	59 5	53 5

Data of Gershenfeld and Witlin, 1941

some of the fatty acids, the alcohols and phenols, the soaps, the dyes, the bile salts, and many of the large number of modern wetting agents and detergents

If the decrease in surface tension were the fundamental cause of death, the bacteria should die when a certain tension is reached, regardless of the chemical structure of the compound used This is not the case Ayers, Rupp and Johnson (1923) found streptococci inhibited at different surface tensions when different depressants were used According to Frobisher (1926), *Staphylococcus aureus* is completely inhibited at a surface tension of 44 dynes per cm when produced by sodium glycocholate, but multiplies readily at as low a tension as 34 dynes when produced by sodium oleate Gershenfeld and Witlin (1941) determined the concentrations required to kill *Staphylococcus aureus* between 5 and 10 minutes, and measured the surface tensions of the killing solutions Table 14 shows that there is no correlation

It may be argued that no direct parallelism between lethal concentration and surface tension can be expected because the lethal effect takes place at the interface between medium and cell membrane, and this interfacial tension need

not be parallel to the tension between medium and air. The interfacial tension between cell and medium cannot be measured. Davis (1927) measured the drop size of oleic acid in chloride solutions, and compared this with the concentration of different chlorides necessary to inhibit bacterial growth. He believed that the toxic effects of the cations can be explained by interfacial tension "if bacteria are looked upon as analogous to oil drops." This analogy has not been generally accepted, and the data presented to support this view show considerable exceptions to the rule.

It must be concluded from these and many other experimental results that death is not caused by change in surface tension, but by some chemical reaction of the surface tension depressant upon some vital cell constituent. However, the subject cannot be dismissed entirely as chemical disinfection because of differences in the mode of action, and of interaction. The difference arises primarily from the concentration of surface-active substances at the surface and interfaces. They act upon the cell not according to the concentration in the liquid, but according to the much higher concentration at the cell surface. This explains perhaps the fact that so many surface-active substances are good disinfectants and antiseptics.

2. *Wetting agents.* The soaps which have been almost the only efficient detergents for more than a thousand years are gradually being replaced by synthetic detergents which decrease the surface tension greatly, but are efficient in acid as well as in alkaline media, and are not precipitated by hard water. These modern "wetting agents" can be divided into anionic, cationic, and undissociated detergents. The composition and origin of a number of these substances is given by Baker *et al.* (1941a, b). In the cationic detergents, the organophilic (or hydrophobic) group is positively charged, as in Emulsol 660 B = (lauryl pyridinium)$^+$ (chloride)$^-$, and in the anionic detergents, this group is negative as in (Na)$^+$ (lauryl sulfate)$^-$. The only neutral, non-ionized detergent mentioned is Demal, representing an organic ester.

Domagk (1935) called attention to the antiseptic properties of this new group of chemical products. According to Baker *et al.*, (1941b) the cationic detergents, as a group, are quite efficient disinfectants, capable of killing gram-positive and gram-negative bacteria in 10 minutes at a concentration of 0.015% (1:6000), or even 0.003%. A few preparations were less efficient. The anionic detergents did not kill these bacteria even after 90 minutes in concentration of 0.1%. No data on surface tension are given.

Cationic and anionic detergents neutralize and precipitate each other. The action of Zephiran is inhibited by decyl sulfate or sodium taurocholate (Baker *et al.*, 1941c) or by Aerosol O T (Gershenfeld and Perlstein, 1943). Different from this ionic reaction is the protection of bacteria against detergents by phospholipids, lecithin and related compounds, if they are added either before or simultaneously with the detergent (Baker *et al.*, 1941c). Addition after the detergent had no effect. Compounds which did not reduce surface tension did not counteract the bactericidal effect. On the other hand, phospholipids did not decrease the germicidal effect of several mercury compounds. Thus,

the germicidal power of detergents and its decrease by other substances is interlinked with a surface tension effect The neutral compound Demal counteracted cationic as well as anionic detergents, it also counteracted gramicidin

Valko and DuBois (1942) found that if a strongly germicidal detergent is mixed with a weak one of the same charge, the total efficiency is smaller than that of the stronger germicide, because the weaker one has occupied many places on the bacterial surface, and the surface concentration of the strong disinfectant is decreased This is an indirect proof for the claim that surface tension as such is not the deciding lethal factor The same authors (1944) also show that bacteria "killed" by a cationic detergent may be revived by addition of a high molecular anion if this is applied within a certain time of perhaps 10 to 30 minutes They consider the reversible death as due to ion exchange in the cell, similar to the death by mercury salts, i e , a chemical phenomenon

The germicidal efficiency of these detergents depends largely upon the pH of the medium in which they act The anionic detergents resemble benzoic or salicylic acid in their rapid increase of efficiency with acidification of the medium (Rahn and Conn, 1944) The analogy permits the conclusion that only the undissociated acid molecules of these compounds have bactericidal properties Gershenfeld and Milanick (1941) found the following concentrations, in ppm, necessary to kill *Staphylococcus aureus* in 5 minutes

	At pH					
	4	5	6	7	8	9
Aerosol OT	29	33	250	>10,000	>10,000	>10,000
Tergitol 4 & 4 T	140	250	500	>10,000	>10,000	>10,000
Triton K 12	>10,000	10,000	3,300	1,250	110	55

The last compound is a cationic detergent, and in this case, the base in its undissociated state is probably the effective agent

3 Combination of disinfectants with surface tension depressants When surface-active substances are added to a disinfectant solution, they may decrease its germicidal efficiency Hampil (1928) obtained such marked inhibition of the bactericidal action of various phenols by the addition of soap as to indicate the impossibility of producing a germicidal soap by incorporating small quantities of a phenolic compound As an example may be given the concentration of butyl resorcinol required to kill *Staphylococcus aureus* between 1 and 2 minutes at different soap concentrations

% Na oleate	0	0 2	0 5	1 0	3 0	5 0
% disinfectant required	<0 05	0 067	0 1	0 2	0 5	0 5

The same has been found to be true with mixtures of mercury compounds and soap, unless the soap is very much diluted (Frobisher, 1927, Rettger *et al* , 1929, Schaffer and Tilley, 1930, Tilley, 1939) The general explanation is that so much soap is concentrated at the surface that the other, stronger disinfectant cannot reach the cell as readily as without soap

It is frequently stated in literature that a decreased surface tension of the medium must increase the efficiency of any antiseptic because the rate of diffusion through the cell membrane is increased For example, Frobisher (1944) points out that aqueous iodine solutions do not spread easily on the skin while an alcoholic solution has a much greater wetting power This leads him to the conclusion in the next paragraph that solutions of disinfectants in dilute alcohol or in similar solvents which have low surface tensions, are much more likely to be effective than when dissolved in water However, Gershenfeld and Miller's data (1932) prove this to be a poor example because with iodine, the difference

TABLE 15

Concentrations, in per cent, required to kill Staphylococcus aureus in 10, but not in 5 minutes

	pH 7	pH 6	pH 5	pH 4
Aerosol concentration when added	0 02	0 008	0 0017	0 0011
Phenol	1 25	1 17	1 11	1–1 05
same + aerosol	0 56	0 42 –0 50	0 33	0.26
Mercuric chloride	0 0067	0 004–0 005	0 004–0 005	0 0025–0 00275
same + aerosol	0 005–0 0067	0 0033–0 004	0 00275	0 0020–0 0022
Merthiolate	0 014	0 014–0 02	0 0056–0 0067	0 0033
same + aerosol	0 0028–0 0033	0 0033	0 0028	0 0020–0 0022
Hexyl resorcinol	0 033	0 025 –0 033	0 013–0 02	0 0067–0 0084
same + aerosol	0 004–0 005	0 004	0 0033–0 004	0 0025–0 00275
Zonite	0 05	0 125 –0 167	0 167	0 25 –0 33
same + aerosol	0 033	0 125	0 125 –0 167	0 20
Zephiran	0 00125–0 00142	0 005	>0 2	>0 2
same + aerosol	>0 2	>0 2	>0 2	>0 2

Data of Gershenfeld and Perlstein, 1941

between aqueous and alcoholic solutions is slight, and in favor of the aqueous solution Frobisher's generalization is erroneous The surface of wood or skin which contains airspaces, is not comparable with the spaces between molecules of a cell membrane which are already completely surrounded by water molecules

However, while not a general phenomenon, the efficiency of *some* disinfectants at *specified* concentrations may be increased by surface tension depressants The increase may be quite noticeable Gershenfeld and Witlin (1941) could find no change in the efficiency of disinfectants by the addition of detergents, but in the same volume, a little later, Gershenfeld and Perlstein did report such an effect (table 15) It is worth noting that at pH 7, Aerosol in concentration

of 0 02% increased the efficiency of phenol 2.2 times, of hexyl resorcinol 7 times, of merthiolate 5 times, of zonite a little, of mercuric chloride not significantly It seems as if only surface-active substances are affected by the presence of wetting agents

This assumption is in agreement with the experiments by Frobisher (1927) who found that a small quantity of sodium oleate (0.25 ml of a 1% solution) increased the efficiency of phenol slightly, while 0 5 ml decreased it greatly Ethyl acetate (0 1 ml) increased the efficiency of phenol and of hexyl resorcinol

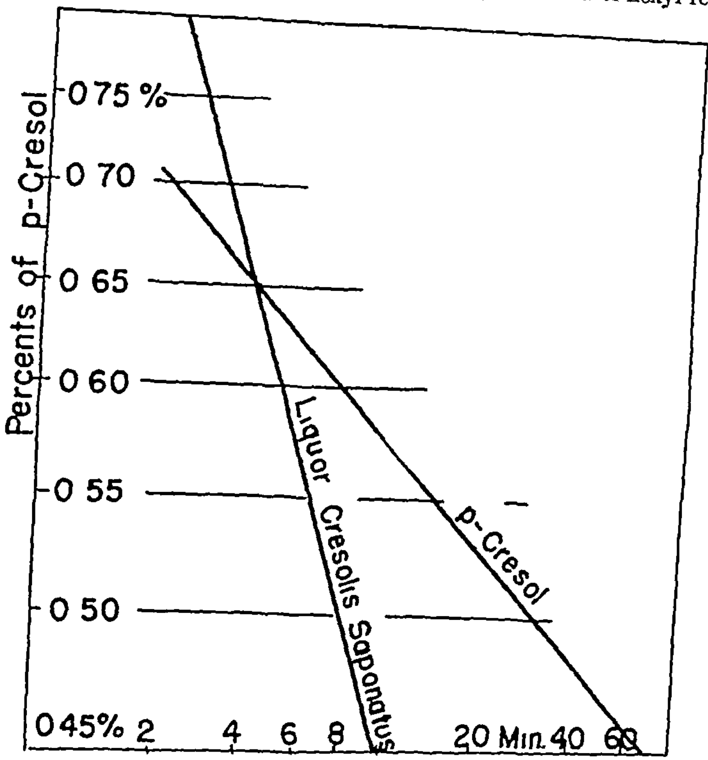

FIG 6 THE EFFECT OF SOAP ON THE GERMICIDAL POWER OF CRESOL LOGARITHMS OF DEATH TIMES PLOTTED AGAINST LOGARITHMS OF CRESOL CONCENTRATION (DATA OF TILLEY, 1939)

considerably All compounds tested decreased surface tension The assumption also agrees with the extensive experiments of Schaffer and Tilley (1930) with mixtures of soap and phenolic compounds The addition of 1 part of soap to 2 of phenol increased the germicidal efficiency considerably Figure 6 from Tilley's later experiments (1939) shows that this increase is limited to a narrow range of concentration In this connection, the following statement of Gershenfeld and Witlin (1941) is of interest "The addition of twelve wetting agents to aqueous solutions of mercuric chloride and arsenic trioxide did not increase the penetration of the inorganic compounds when applied to the unbroken skin of rabbits " Mercury and arsenic compounds do not affect surface tension greatly

4 Bile solubility Another phenomenon usually ascribed to surface tension depression is the bile solubility of certain bacteria, notably pneumococci Höber and Höber (1942) treat this solubility in a general way, although they are discussing primarily blood cells They describe the action of bile salts and detergents as follows

"Their molecules are composed of two portions, one polar hydrophilic, which has a tendency to anchor the molecule to water, and one nonpolar hydrophobic which is attracted towards the nonaqueous phase The result is a molecular orientation at the interface An increase of hydrophilic affinity will lead to a stronger pull on the nonaqueous phase toward the water and may have a disintegrating or a dispersing effect Stronger organophilic properties on the other hand will favor a wetting action, *viz* , the fixation of a film of water on the nonaqueous phase or its separate components Regarding especially the interface between a cell and its surroundings, hydration, swelling and dissolution can be due to the wetting action on the colloidal structures or the micellae Protein molecules are unfolded or disrupted by the pull, and thus denatured In conjugated proteins, the bonds between the protein part and the prosthetic group are severed, viruses and enzymes are inactivated, and the final result of such effects on cells is cytolysis "

This phenomenon of complete dissolution by such compounds as bile and bile salts is not caused by the decreased surface tension as such, for other depressants like the alcohols, phenols and saponin do not dissolve bacteria Of special interest in this differentiation between dissolving and non-dissolving agents is the report by Falk and Yang (1926) that sodium oleate dissolves washed pneumococci when re-suspended in distilled water, but not when re-suspended in 0 85% NaCl solution Leonard and Feirer (1927) reported that *Endamoeba coli*, *Iodamoeba williamsi*, *Leptospira icterohaemorrhagiae*, *Trypanosoma lewisi*, and *Trichomonas hominis* are instantly destroyed and disappear completely on contact with hexyl resorcinol solutions while bacteria do not The reviewer has observed that cationic detergents did not dissolve pneumococci at pH 9 8 where they were most efficient as germicides While germicidal power depends primarily on the concentration of the undissociated base, dissolving power depends upon other properties

Our knowledge of the interaction between surface tension depressants and bacteria is not at all perfect or complete The sterilizing properties of the compounds of this group are certainly not due to the decreased interfacial tension as such However, the intensity of the chemical reaction of these compounds is influenced by their effect upon interfacial tension, and they may, by this effect, increase or decrease the germicidal power of other disinfectants

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RELATION OF BACTERIA TO VITAMINS AND OTHER GROWTH FACTORS

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CONTENTS

Seven years ago the subject of growth factors was reviewed by Koser and Saunders (167) for this publication Since that time, great progress has been made in the field At that time only seven compounds could be listed as accessory growth substances for bacteria Today the list of compounds has expanded to about twenty Five of the additions are also new animal vitamins, but eight have not yet found a place in animal nutrition The merging of the fields of animal and microbiological nutrition is well illustrated by the fact that three of these vitamins (biotin, pantothenic acid, and *para*-aminobenzoic acid) were discovered through microbiology, while the other two (pyridoxine and choline) appeared first in the field of animal nutrition It is probably safe to predict that several of the compounds now known to be required by bacteria will some day become members of the vitamin family A mutual exchange of ideas is bound to promote progress in both fields, and the benefits that accrue from such an exchange indicate the artificial nature of the boundaries that divide research work on cellular nutrition

In contrast to the previous review, this paper will be limited to growth factors

for bacteria and the literature covered is that which appeared through 1944 A few references appearing in 1945 have been cited but no systematic effort has been made to review papers published during the present year The developments in the field of bacteria alone have been so extensive during the last six years that it is difficult to cover the subject adequately in a review of reasonable length Growth factors as used in this paper are defined as organic compounds that must be present in the medium in only minute quantities to promote the development of bacteria The purpose of this limitation is of course to exclude the mineral elements and well-defined organic compounds such as amino acids In most cases the limitation "minute amounts" is a sufficiently sharp criterion, but at times it becomes a narrow and almost vanishing boundary For example, *Lactobacillus casei* requires only about twelve times as much tryptophane (352) as nicotinic acid (188) The nicotinic acid requirement, on the other hand, is a thousand fold that for biotin The exclusion of the amino acids from the review logically required the omission of glutamine and asparagine in spite of the prominent place these two compounds occupy in growth-factor work Whether this prominence rests on something other than amino-acid characteristics does not seem clear at the present time

Substances that are not required preformed in the medium but if added increase the rate of growth are included in this review, as well as those that are required for the initiation and maintenance of growth There are several reasons for not excluding the stimulatory substances A compound that is indispensable in one medium may be unnecessary or only stimulatory in another (396, 449) Again, the microörganism that at first required the compound in the medium may be trained to grow without its addition (169, 361) It is probable that in such cases the microörganism has become able to synthesize as much of the compound as it needs for its metabolism The success that has been attained in the adaptation of bacteria to dispense with various amino acids shows the remarkable latent powers of bacteria and suggests the possibility that similar success may be reached in training bacteria to dispense with growth substances

RESPONSE OF BACTERIA TO GROWTH SUBSTANCES

Table 1 lists various compounds and unknown factors that have been reported to promote the growth of one or more species of bacteria Numbers in the blocks refer to papers reporting a favorable effect Where there is no entry, no report on the use of the compound has been found If the compound has been tried and found to have no effect this result is indicated by the word "no" A statement regarding the manner in which this table has been compiled may be helpful to the reader

No distinction is made between compounds that are required in the medium and those that are merely stimulatory to growth, because it is often impossible to distinguish between them For example, Wood, Andersen and Werkman (447, 449) showed that a given strain of propionic acid bacteria required riboflavin in an ammonium sulfate medium but not in an amino acid medium They also demonstrated that strains of propionics could be adapted by subculturing to dispense with riboflavin and thiamine

Likewise, no attempt is made to distinguish between different strains of the same species with respect to their requirements Some strains require a certain compound in a given medium and other strains do not Difference of strains is one explanation for the seeming contradiction of an organism being listed as both requiring and synthesizing a growth factor

Growth factors that have not been isolated are listed in the last column under the heading of unknown factors, if the reported factor appears to be distinct from the known compounds listed in the other columns In case of papers published several years ago, it is difficult to decide whether or not a new factor was involved, as some of the compounds now known to promote the growth of bacteria were unknown or unavailable at the time In connection with unknown factors the terms, Bios I, IIA, IIB, etc are not used in this review as they have little meaning at the present time In the field of the vitamins, the letters B_1, B_2, B_6, etc are being dropped and the corresponding chemical terms thiamine, riboflavin, pyridoxine (pyridoxal, pyridoxamine), etc are being substituted Numbers or letters serve as suitable and temporary means of designation and also have the advantage that they can be readily abandoned when the pure compound has been isolated, identified, and properly named On the other hand, to give chemical names to substances that have not been obtained in pure form appears also to be undesirable and unsound Such terms are likely to be inappropriate and to carry a meaning which they do not legitimately possess Their introduction is also unfair to other investigators in the same field who seek to avoid cluttering the literature with premature and inappropriate chemical nomenclature

The bacteria as named by the authors cited are listed in alphabetical order This procedure results in the listing of organisms that are regarded by some authorities as identical under two different names Since there is disagreement among investigators as to the proper name to be applied, and since in some cases the name in general use is not that of systematic taxonomy, it would make the review confusing and less useful to list the bacteria under names other than those in current use

Conclusions drawn from table 1

Approximately 130 bacteria have been found to need one or more of the compounds listed in the table Organisms that are known to require the largest number of compounds are *Clostridium tetani*, *Lactobacillus arabinosus*, *Lactobacillus casei*, pneumococci, *Streptobacterium plantarum*, hemolytic streptococci and *Streptococcus lactis* with requirements of from 7 to 10 each It is not implied, of course, that other bacteria do not utilize just as many growth factors as do the microörganisms listed Probably all of these compounds are involved in bacterial metabolism, but when an organism is able to synthesize unsupplied factors their presence in the medium is not required This aspect of bacterial nutrition will be dealt with in a later section

Only a small proportion of the total number of heterotrophic bacteria have had their complete requirements for growth substances worked out There is great need for systematic work to fill in the gaps in our knowledge, but with the

TABLE 1

Index of growth factor requirements of bacteria

Key to Table The bacteria are listed in column one and the growth factors required by the bacteria are designated by numbers in the successive columns The numbers refer to the bibliography and denote that the compound is reported as being required or stimulatory for the growth of the corresponding organism in column one No attempt is made to distinguish between different forms of a growth factor, *e g* nicotinic acid *vs* the amide, pyridoxine *vs* pyridoxal

The letter "S" means that the organism is reported to synthesize the growth factor The reference to the synthesis is given in table 3

The word "no" followed by a number signifies that the organism has been found not to require the growth factor preformed in the medium

Where there is no entry in a block, it means that no report has been found regarding the need, synthesis, or dispensability of the compound for the corresponding organism

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
Acetobacter suboxydans	S	138, 185, 417	185, 417		185, 189, 417	S		Butyrolactone, 417, purines, 195	
Bacillus brevis	190								
B butylicus	183								
B dextrolacticus						6	6		Unnamed, 6
B larvae	No, 204	No, 204	No, 204	No, 204		No, 204	204	Inositol, no, 204	
B macerans	139						139		
B mesentericus	48	S				S	S		
B polymyxa	139								
B proteus		93b, 148, 214, 215							
B saccharobutyricus	183								
Bacterium bifidum						202, 294			

Bacterium brassicae			249, 384, 385			No, 381			
Bacterium radicicola (See *Rhizobium*)									
Bacterium tularense	407	407	407						Liver, etc., 407
Bacterium utile				253, 255					
Bacterium typhosus									"Sporogenes vitamin," 94
Betabacterium breve						202, 204			
Brucella abortus	163, 168, 218, 219, 220	141, 163, 168, 219, 220	163, 168, 219, 220		S		141, 163, 168, 218, 219, 220		
Brucella melitensis	220	163, 220	163, 220				163, 218, 220		
Brucella suis	220	163, 218, 220	163, 218, 220				163, 218, 220	β-Alanine and butyrolactone, 163	
Clostridium acetobutylicum	182, 183, 207a, 300, 432				123, 182, 183, 184, 300, 334, 335	S			"Acetone factor," 335
C beijerinckii							No, 46a		Yeast, 46a
C botulinum	100				S				"Sporogenes vitamin," 93a, 155, unnamed, 121b

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
C butylicum	181, 183, 309a, 386	S	S	S	183	S	S		
C chauvoei	100				183				
C felsineum	183								
C histolyticum	100								"Sporogenes vitamin," 86
C parabotulinum							No, 16a		Yeast, 16a
C pectinovorum							20		Hydrolyzed casein, 20
C septicum	20	20		20					
C sporogenes	183, 309a				S				"Sporogenes vitamin," 155, 208
C thermosaccharolyticum	63	63	63		63		63		
C tetani	90, 91, 183, 272, 273	91	90, 91, 272, 273	91	S	90, 91, 272, 273, 314	90, 91, 272, 273	Purines, 90, 91, 272, 273, oleic acid 90, 91, uracil, 90, 91	Eluate factor, 272, "folic acid," 90, 91, 273, 271
C welchii	100			91				Uracil, oleic acid, 91	
Corynebacterium diphtheriae	120	12, 266, 267, 268, 269	271		S	S	S	β-Alanine or pantothenic acid, 166, 268, 270, oleic acid 65, 66, pimelic acid or biotin, 263, 264, 265, 120	Blood factor, 389, liver factor, 57, 59

C diphtheriae (*intermedius*)		88, 89	88, 89				S	β Alanine or pantothenic acid, 88, 89, pimelic acid, 88, 89	Liver factor, 53, 58
C diphtheriae (*mitis*)		88, 89	88, 89			S	S	Pimelic acid, 88, 89, β-alanine or pantothenic acid, 88, 89	Liver factor, 55, 58
C diphtheriae (*gravis*)		88, 89	88, 89		55	S	S	Pimelic acid, 88, 89, β-alanine or pantothenic acid, 88, 89, oleic acid, 66	Liver factor, 55, 58, blood factor, 66
Dysentery bacilli (See *Shigella*)									
Erysipelothrix rhusiopathiae						127		Oleic acid, 127	Unnamed, 127
Escherichia coli	S	S, 331*				S	S, 331*	Indole acetic acid, 13	
Gonococcus see *Neisseria gonorrhoeae*									
Hemophilus canis								Hematin, 208, 329a	
Hemophilus ducreyi								Hematin, 213a	

* X ray treated strains are reported to require these factors

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
H influenzae								Hematin (factor X), 18, 71, 72, 93c, 103, 104, 208, 213, 291, coenzyme I (factor V), 18, 208, nicotinamide riboside, 104b	Unnamed, 18
H parainfluenzae								Coenzyme I 18, 160a, 208–212, 261, 310, nicotinamide riboside, 104b, 343	Unnamed, 18
H pertussis		121a							
Klebsiella pneumoniae		472							
Lactobacillus arabinosus	387, 466	384, 385, 387	240, 384, 385	S	131, 377	S, no, 381		Purines, 376, 377, pyrimidines, 376	Folic acid, 21 Tomato factor, 180a
L acidophilus	190					51			
L beijerinckii						51			
L bulgaricus						51		Thymine, 304	Folic acid, 304

L casei	188, 353, 354, 395, 416	188, 384, 385	188, 240, 308, 384, 385	37, 38, 188, 370, 371, 372, 379	54, 62	51, 188, 380, 381, 382, 383	21	Purines, 92, 399, thymine, 174, 394, 399, orotic acid, 52, fatty acids 20, 402	Eluate factor, 124, 378, 379, unnamed, 399, 400, folic acid 250, 252, vitamin B_c, 31, 309, *L casei* factor, 125, misc, 54, 64, 76, 85, 92, 392
L delbrückii			384, 385	37		51, 380, 381		Thymine, 394	Eluate factor, 124, folic acid, 394
L gayonii						381			
L helveticus						51			
L jugurt						51			
L lactis			240, 384, 385	37, 38, S		51, 175, 380, 381			
L leichmanii						51			
L lycopersici						447			
L mannitopoeus						447, no, 381	358, 360		
L pentoaceticus						No, 381, 447			
L pentosus			384, 385		377	No, 381, S		Purines, 376, 377, pyrimidines, 376	
L plantarum						No, 51			

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
Leptospira canicola		333a				333a	333a		In animal serum, 333a
L hebdomadis icterohaemorrhagiae		472							
L icterohaemorrhagiae		426, 472							
Leuconostoc mesenteroides	99, 100	99, 100	99, 100, 384, 385	37, 99, 100		No, 381	99, 100	Purines, 376, 377, no, 99, pyrimidines, 376, no, 99	
Listerella monocytogenes	128, 317					127, 317	128, 317	Hemin, 317	Casein factor, 127, 128
Mycobacterium paratuberculosis								Anti hemorrhagic compounds, 460	
M phlei		472						Ergosterol, 320	
M tuberculosis								Ergosterol, 320	Liver, etc , 11
Neisseria gonorrhoeae		434?	134?	134?			434?	Cocarboxylase, 106, choline, 434	Liver, etc , 5, 107, 134
Pasteurella pestis	No, 84	328 No, 84	No, 84	No, 84	No, 84	No, 84	328 No, 84	Hematin, 328, no, 84	
P suiseptica		25, 27, 28, 162	25, 27, 28						Unnamed, 25

P tularense							25, 27		
Photobacterium phosphorescens						83			
Pneumococci (several types)	12, 39, 104a	12, 104a, 326, 327	12, 104a, 326, 327			326	12, 104a	Purines, 104a, choline, 12, 104a, 326, 327	Strepogenin, 455
Propionibacterium arabinosum			170			448	408, 448		Unnamed, 412
P jensenii	412	No, 412	170, 412	No, 412	412	No, 412		Inositol, no, 412	
P pentosaceum			170, 384, 385			449	356, 358, 360, 408, 448, 449		Eluate factor, 124, unnamed, 412
P petersonii	412	No, 412	412	No, 412	412	449, no, 412	356, 358, 412, 449	Inositol, no, 412	
P rubrum	412		412	412		449	449		Unnamed, 412
P shermanii			170						
P technicum		No, 412	412	No, 412		No, 412		Inositol, no, 412	
P thoenii	412	No, 412	170, 412	No, 412		449, no, 412	449	Inositol, no, 412	
P zeae			170			S	408, 449		Unnamed, 412
Proteus morganii	No, 305	302, 303, 305	26, 78, 120, 302, 303, 305	No, 305	No, 305	No, 305	No, 305	Inositol, β-alanine, pimelic acid, purines and pyrimidines, no, 305	Unnamed, 305
P vulgaris	S	93b, 214, 257, 260, 301, 336	S	S	S	S	S	*Inositol*, S	

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
Pseudomonas aeruginosae pyogenes	S	No, 341			S				
Rhizobium leguminosarum	277, 440, 446						No, 277		Unnamed, 61
R lupinii	439, 440								
R meliloti	440, 446								
R phaseoli	440								Unnamed, 61
R trifolii	281, 282, 283, 438, 439, 440, 446, S		440			437a, 439, S	33, 34, 270, 280, 281, 282, 283, 437a S	β Alanine, 440	
Rhodospirillum rubrum	120							Pimelic acid, no, 120	
Salmonella gallinarum							135, 136, 137		
S paratyphi		111, 145, 146, 147, no, 341			S				
S pullorum	No, 136	135, 136, 137	No, 136	No, 136		No, 136	No, 136		
S schottmuelleri		No, 341							

Sarcina flava						340	340		
Shigella dysenteriae		114 to 148			S				
S paradysenteriae		80, 81, 82, 96, 162, 161, 165, 169, 336, 130, 472	430	No, 165	S			Uracil, 130	
Shigella sonnei		81, 161, 165		No, 165					
Spirillum serpens						S		Purines, 306	
Spironema gallinarum						No, 151	No, 151	Pimelic acid, no, 151	Chicken red cells, 151
Staphylococcus albus		166		422	S	S	166, 305		
S aureus	315, 316, S	140, 152, 153, 154, 156, 160, 186, 315, 316, 435			S	S	119, 149, 153, 154, 156, 315, 316, 305,	Uracil, 320	Unnamed, 121b
S pyogenes aureus	160, 176, no, 339	160, 176, 339, no, 311					160, 176, 339	Inositol, no, 339	
Streptobacterium casei						202, 203, 205			
S plantarum	176, 254, 257, 259	176, 254, 257, 259	176, 254, 259	176, 253, 254, 255, 259	176, 250	250	176, 254, 255	Purines, 176, 251, 255	
Streptococci (hemolytic)		325	223, 224, 405	224	S	325		Betaine, 325, thiochrome, 325	

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC	UNKNOWN
Streptococci (hemolytic) Group A	122, 299	299	30, 209	290		209	299	Purines, 299	Strepogenin, 392, 455, unnamed, 10,110
Streptococci (hemolytic) Group B	284	284	284, 459	284, 459		284, 450	284		
Streptococci (hemolytic) Group C			458			458		Pyrimidines, 332	
Streptococci (hemolytic) Group D			453, 459	346, 459		459		Butyrolactone or pantothenic acid, 453	Strepogenin, 392
Streptococci (hemolytic) Group G			223						
Streptococcus bovis	287, 363	287, 363				S	287, 363		
S *cremoris*	285	285	170, 285	285		285, 292, 295	285		
S *distendens*			170						
S *durans*	286	286	286	286		286	No, 286	Thymine, 394	Folic acid, 286, 394, SLR factor, 397
S *epidemicus*			458			458			
S *faecalis*	286	286	286, 346	286, 346		51, 286, 346	No, 286	Thymine, 394	Folic acid, 286, 394, unnamed, 464, SLR factor, 397

S lactis ATCC 8043*	207, 248, 376	207, 218, 376	207, 248, 219, 383, 384, 385	207, 218, 370, 371, 372		No, 381, S		Purines, 207, 376, 377, thymine, 171, 376, 394, alanine or pyridoxine, 369	Folic acid,† 207, 218, 250, 252, eluate factor,† 121, *L casei* factor,† 125, 399, 100, vitamin B_c, 31, 171, 309, 410, factor SLR,† 142, 307
S lactis	285	285, 321	285	285		292, 295, yes and no, 285, 324	Yes and no, 285	Purines, 324	Unnamed, 361, 464, strepogenin, 392
S liquefaciens	286	286	286	286		286	No, 286		
S mastitidis			170, 459	159		459, no, 51			
S paracitrovorus						447			
S pyogenes			158			458			
S salivarius	287, 303	287, 363	287, 363			287, 363	287, 363	Uracil, 287, 303	
S thermophilus						292, 295	118		
S zymogenes	286	286	126, 286, 159	126, 224, 286, 459		51, 126, 286, 459	No, 286	Thymine, 391	Folic acid, 286, 391, factor SLR, 307
Thermobacterium bulgaricum						202, 205			
T helveticum						2, 202, 205			Unnamed, 423
T jugurt						205			
T lactis						203, 204, 205			

* According to Niven and associates (116, 285, 286), this organism should be classified as *Streptococcus faecalis*
† These factors are replaceable by thymine (171, 391)

accelerating rate of progress that is now being attained, another five years should close most of the gaps for the commonly used bacteria Even though we know what compounds will promote growth for an indefinite number of transfers, it by no means follows that we have discovered the requirements for optimum growth and metabolism Comparison of the defined medium with the best natural medium often shows that the former gives a slower rate of cell proliferation and formation of products than the latter When equivalence has been reached, we may attempt to improve on nature Today we are undoubtedly short of equivalence in most cases

The compounds most frequently reported as promoting the growth of bacteria are biotin, nicotinic acid, pantothenic acid, and riboflavin, each being required by about 50 organisms, thiamine 40, pyridoxine (pyridoxal, pyridoxamine) 25, *p*-aminobenzoic acid 15, about 15 each for several purines and pyrimidines, and 5 or less for more than a dozen other compounds Some compounds, such as ascorbic acid and thioglycolic acid have been reported (9, 12, 49, 104a, 150, 216, 340, 458) as favoring the growth of certain bacteria, but it is probable that they do so by regulating the oxidation-reduction condition of the medium rather than as true growth substances Because of this doubt regarding the function of these compounds, they have not been listed in the table

Many of the above compounds are well-known vitamins for animals, but there are many compounds having potency for bacteria that have not yet found a place in the animal field The opposite is of course also true, most of the fat-soluble vitamins are not known to have any potency for bacteria A pair of compounds of great potency in the growth of higher plants is conspicuously absent from the lists of both animal and microbiological vitamins, *viz*, auxins a and b Their occurrence in plant and animal tissues, *e g*, seeds and liver, strongly suggests that they play an important role in the metabolism of such cells It is not improbable that some day they will be found to serve as growth factors for bacteria

Unidentified factors

The number of bacteria listed in table 1 as requiring unknown growth factors is about thirty It seems unlikely that there is any such number of unknown factors The requirements of these bacteria can probably be met in most cases by known compounds that were either not available or were not recognized as growth factors at the time the papers were published In other cases, failure to grow may have been due to inadequate supplies of amino acids and mineral elements or to unsatisfactory buffer and oxidation-reduction conditions rather than to growth factor deficiencies There remain, however, several instances in which the existence of an unidentified factor seems well-established, and other cases where its occurrence seems strongly indicated These will be considered in order

Sporogenes vitamin This is the oldest unidentified factor and was first described by Knight and Fildes (155) Pappenheimer (298) made an extensive chemical investigation of its concentration and properties His best preparation,

although not crystalline, was very potent 0 04γ per ml of medium insured good growth of *Clostridium sporogenes* The preparation was acidic in nature, very stable to heat and acids, and had an apparent molecular weight of about 200 In many respects, the data indicate a strong resemblance to biotin, but in other respects they show a marked unlikeness, notably in the absence of nitrogen and sulfur from the preparation Further indication that biotin is probably involved in the action of the sporogenes vitamin comes from the reports of Peterson, McDaniel and McCoy (309a) and Lampen and Peterson (183) that certain strains of *C sporogenes* require only biotin for growth on a synthetic medium although other strains fail to grow on this medium Only biotin is required by several clostridia, but thiamine, riboflavin, pantothenic acid and *p*-aminobenzoic acid are required by others On the basis of available data, it appears probable that "sporogenes vitamin" consists of several known factors but may also contain some as yet unidentified factor A systematic study employing the known compounds would undoubtedly throw much light on the problem

Norite eluate factor, Lactobacillus casei factor, vitamin B_c, folic acid, SLR factor In 1939 Snell and Peterson (378) published an abstract and in 1940 a complete paper (379), concerning the occurrence, concentration, and properties of a growth factor required by *Lactobacillus casei* [1] The factor was abundant in liver, malt products, and yeast, and occurred to a less extent in an extract of cereal grains Adsorption on norite and elution therefrom was one of the most effective means of concentration, and because of this property, the factor was designated "norite eluate factor" In 1941, Hutchings *et al* (124) published further details regarding the purification and properties of the factor and showed that it was required by several other lactic and propionic acid-forming bacteria, *e g*, *Streptococcus lactis* R, *Propionibacterium pentosaceum*, but could be synthesized by certain lactic types, *e g*, *Lactobacillus arabinosus*, *Leuconostoc mesenteroides* In the same year, Stokstad (399) also reported the preparation of an active concentrate from liver which could be replaced in part by thymine

In 1941 Mitchell, Snell, and Williams (250) published a brief note reporting the concentration from spinach of a factor required by *Streptococcus lactis* R (No 8043 of the American Type Culture Collection)[2] but also potent for *L casei* Because it was considered to be "a nearly pure chemical entity" (later found to be about 30% pure) and because of its abundance in leaves, the factor was named "folic acid"

The two terms, "norite eluate factor" and "folic acid" obviously referred

[1] This organism is widely used in microbiological assays and at various times has been called *L casei*, *L casei* ϵ, and *L helveticus* On the basis of a recent bacteriological report (414) the most suitable name is *L casei* It is carried under the serial number 7469 by the American Type Culture Collection, Georgetown University Medical School, Washington, D C The discussion that follows applies only to this strain of *L casei* Other strains of *L casei* may not have the same growth-factor requirements as Culture 7469

[2] Attention is called to the recent reports of Niven and Sherman (286), Niven (285), and Gunsalus, Niven, and Sherman (116) stating that *S lactis* R is really a strain of *Streptococcus faecalis* The designation *S lactis* R is retained in conformity with the terminology used in the original papers

originally to the same factor or factors but with some special emphasis in each case on properties, occurrence, or test organism Other investigators have shown that these factors are required by several additional bacteria

In a series of four papers, Mitchell *et al* (97, 245, 251, 252) reported in detail a procedure for the concentration of folic acid, and supplied much information regarding its chemical and physiological properties On the basis of elementary analysis and molecular weight determinations (400 ± 50) a formula of approximately $C_{15}H_{15}O_8N_5$ is indicated

Interest in these factors was greatly stimulated by the discovery that they were involved in animal nutrition Hutchings *et al* (123a) reported that the norite eluate factor was required for the growth of chicks Since that time a large number of reports showing its importance for rats, chicks, and monkeys have appeared A discussion of these reports is outside the scope of this paper, but they have been adequately covered in a recent review by Wieder (441)

The converging lines of investigation in microbiological and animal nutrition met in the isolation from liver of a crystalline compound by Pfiffner *et al* (309) in 1943 This compound was potent in extremely small quantities for both the chick and *L casei* Because the initial research arose in pursuit of a chick antianemia factor which had been named vitamin B_c, this term was also used to designate the *L casei* factor The compound was obtained as yellow or orange colored crystals that in the most recently published analysis (Binkley *et al*, 31) had the following percentage composition C 52 45, H 4 29, N 19 7

The announcement of Pfiffner *et al* was soon followed by a note (Keresztesy *et al*, 142) reporting the isolation[3] from an undisclosed source of a very potent factor for *S lactis* R (later called SLR factor) but almost inactive for *L casei* In a later report, Stokes *et al* (397) gave the interesting information that *S lactis* R and other streptococci of the enterococcus group can convert the SLR factor into a form which is active for *L casei* and other lactobacilli Factor SLR is regarded by its discoverers as different from folic acid because of the lack of response of *L casei* to it but, since folic acid is defined (Snell and Mitchell, 376, and Mitchell and Williams, 252) as the factor required by *S lactis* R, there is obviously an overlapping of territory in these two terms

A few months later in 1943, Stokstad (400) announced the isolation of crystalline compounds from both liver and yeast that had essentially the same composition and properties as vitamin B_c Stokstad's compounds had equal potency for *L casei*, but for *S lactis* R the yeast product was only about one-half as potent as the liver isolate A fourth crystalline compound derived from still another source was reported from the same laboratory by Hutchings *et al* (125) This compound had about the same spectral absorption as the liver and yeast compounds but differed from that of highly purified folic acid It was about 80 to 90 per cent as active for *L casei* but only about 6 per cent as active for *S lactis* R as the liver product

A still further complication in the picture is brought about by the discovery

[3] The original report did not say whether or not the compound was crystalline but in a private communication Dr Keresztesy states that it was obtained in crystalline form

that, in yeast, vitamin B_c also occurs in the form of a conjugate that is highly active for the chick but has little potency for either *L casei* or *S lactis* R However, on digestion with an enzyme found in kidney and other animal tissues but not in yeast, Bird *et al* (32) found that the conjugate becomes highly active for both organisms From the digestion material a crystalline compound was isolated identical in properties and potency with the crystals from liver Another report on the possible existence of a "folic acid"-like material inactive for bacteria but having vitamin activity for the rat has been given by Welch and Wright (433) At the time this review is being written (January 1945), there appear to be a pure compound that is very active for both organisms, a second that has the same activity as the first for *L casei* but only one-half the activity

TABLE 2

Source and activity of crystalline or highly purified compounds required by Lactobacillus casei (A T C C 7469) and Streptococcus lactis R (A T C C 8043)

PREPARATION	NAME	SOURCE	TEST ORGANISM	AMOUNT FOR ½ MAX ACTIVITY	INVESTIGATOR
				$m\gamma/ml$	
1	Vitamin B_c	Liver	*L casei*	0 05	Pfiffner *et al* (309)
	Vitamin B_c	Yeast	*L casei*	05	Binkley *et al* (31)
	Vitamin B_c	Liver	*S lactis*	25	Hutchings *et al* (125)
	Vitamin B_c	Yeast	*S lactis*	50	Stokstad (400)
2	Factor SLR	Undisclosed	*S lactis*	034	Keresztesy *et al* (142)
	Factor SLR	Undisclosed	*L casei*	20 had no effect	Keresztesy *et al* (142)
3	*L casei* factor	Undisclosed	*L casei*	061	Hutchings *et al* (125)
	L casei factor	Undisclosed	*S lactis*	4 2	Hutchings *et al* (125)
4	Folic acid	Spinach	*S lactis*	055*	Mitchell and Snell (248) Mitchell and Williams (252)
	Folic acid	Spinach	*L casei*	072*	Snell (369)

* Calculated for folic acid of 137,000 potency

of the liver product for *S lactis* R, a third that is active for *L casei* and almost inactive for *S lactis* R, a fourth that is just the reverse of the third, and a fifth, as yet not isolated in a pure state, that is inactive for both organisms

The similarities and differences among the various compounds can perhaps be most readily seen from the brief tabulation set forth in table 2 Many other papers dealing with the testing and potency of these compounds have appeared, but so far no one group of workers has tested all of the pure compounds Comparisons of potency are hence difficult to make, but vitamin B_c appears to be the most potent for *L casei*, and factor SLR the most potent for *S lactis* R The potencies reported place these compounds among the extremely active growth factors, of which biotin is perhaps the best example

It is probable that these compounds possess some unit structure in common Those compounds for which most information is available, *viz*, vitamin B_c, *L casei* factor, and folic acid, contain a carboxyl group The methyl ester is inactive but much of the potency of the free acid can be recovered after saponification of the ester The *L casei* factor appears to contain a free amino group as it becomes inactive on treatment with nitrous acid, on acetylation, or on benzoylation *L casei* factor and folic acid have been related to xanthopterin because of a similarity in ultraviolet absorption spectra (Stokstad 400, Hutchings *et al*, 125, Mitchell, 245, and Bloom *et al*, 36) and hence it seems probable that the several factors contain a unit structure similar to that of xanthopterin Additional evidence of a relationship to xanthopterin is found in certain common physiological properties cure of fish anemia, relation to one another in synthesis or destruction by rat liver, and inhibition of synthesis by *Aerobacter aerogenes* (Wright *et al*, 465, 467–469)

Although *L casei* can be grown in continued subculture in a medium containing only crystalline compounds (174, 416), unidentified factors that stimulate initial growth have been reported by Feeney and Strong (92, 93), Pollack and Lindner (313), Light and Clarke (202), Dolby *et al* (76), and Sprince and Woolley (392), but it is not clear how much of the effect obtained is beyond that resulting from the addition of increased amounts of known compounds, *e g*, amino acids and vitamins Thus Feeney and Strong could replace their yeast extract with a mixture of compounds of which asparagine and glutamine were the most effective Pollack and Lindner found glutamine was ten times as potent as peptone, the source of their factor, and Chu and Williams (62) report that when glutamine, pyridoxal, and *p*-aminobenzoic acid were added to the basal medium, peptone had no effect Snell (369) found that 0 5% hydrolyzed casein, the amount usually used in the basal media, does not supply sufficient alanine, and Dolby and Waters (77) obtained increased growth on the addition of leucine, isoleucine, and threonine Lowry and Bessey (206) discovered that making conditions more anaerobic, *e g*, by addition of cysteine to the medium and replacement of air with CO_2, markedly improved acid production and presumably growth of *L casei* The size of the inoculum and the way it was prepared also appear to be of importance in determining the effect of stimulating substances Besides a factor that stimulates initial growth, Dolby, Happold, and Sanford postulate two other unknown factors for *L casei*, but they do not appear to have excluded the several compounds recently isolated in crystalline form by American investigators

The situation that exists with respect to *L casei* appears to be repeated with *S lactis* Smith (364), Sprince and Woolley (392), and Wright and Skeggs (464) report growth-promoting effects with unidentified factors obtained from yeast, liver and casein Niven (285), on the other hand, tested 21 strains of *S lactis* and all grew in 24 hours in a defined medium provided sufficient unheated filter-sterilized glutamine and asparagine were added to the autoclaved medium When these compounds were autoclaved in the medium, the response was erratic Pollack and Lindner (312) and Wright and Skeggs (464) suggest that

asparagine or glutamine may be involved in the structure of proteins or in the synthesis of compounds which are more active for the bacteria than the original compounds

Factors for other lactic acid bacteria Orla-Jensen *et al* (294, 295) have reported that unidentified growth factors contained in milk are required for various lactic acid bacteria Some of the effects they obtained from "milk bios" were probably due to *p*-aminobenzoic acid and to the recently isolated compounds that promote the growth of *L casei* and *S lactis* R

Möller (254, 255) reported that three unidentified factors, G, H′, and J, were required by *Streptobacterium plantarum*, but apparently these have been replaced by known compounds, for in later papers Möller and Schwarz (259) and Kuhn and Schwarz (178) state that H′ is *p*-aminobenzoic acid, and that the organism can be grown in media containing only known crystalline compounds In a later paper, Kuhn *et al* (176) give the composition of the synthetic medium, this consists of 26 very carefully purified compounds (glucose, amino acids, salts and growth factors) An acid-stable factor found in tomato juice that stimulates the growth of *Lactobacillus arabinosus* has been reported by Kuiken *et al* (180a)

Corynebacterium diphtheriae factors A group of English investigators (Chattaway *et al*, 53, 55, 57, 58, 59) have published several notes and papers regarding unknown factors required by *intermedius*, *mitis*, and *gravis* strains of the diphtheria bacteria The factors appear to be closely related to one or another of the recently isolated *L casei* and *S lactis* R factors Differences or identities can be established only when pure compounds have been tested

Factors for Erysipelothrix and Listerella Hutner (127) reported that *Erysipelothrix rhusiopathiae* required an unidentified factor found in yeast and peptone, that was partly adsorbed by fuller's earth and completely adsorbed by charcoal In the same paper Hutner dealt with the requirements of *Listerella monocytogenes* and found that it required an unknown factor also found in yeast and peptone and similar in properties to the erysipelothrix factor In a later abstract, Hutner (128) states that this factor is not folic acid, xanthopterin, oleate, or acid-hydrolyzed yeast nucleic acid The factor is found in so-called "vitamin-free" casein and withstands acid hydrolysis

Gonococcus factors A thermolabile factor required by certain fastidious strains of *Neisseria gonorrhoeae* has been identified by Lankford and Snell (198) as glutamine, but all strains tested by Lankford *et al* (197) required one or more thermostable factors found in liver and other tissues Certain other exacting strains required cocarboxylase in addition to the unidentified factors (Lankford and Skaggs, 196) This requirement for cocarboxylase recalls the relationship between nicotinic acid and coenzymes I and II with respect to the requirements of the hemophilus bacteria Gould (105) found that glutathione is essential for certain strains of gonococci after they have been cultured in the laboratory for a few weeks When freshly isolated, they did not require glutathione This is an example of an induced requirement, a phenomenon that is much less common than a relinquished requirement Gould also reported that, by suitable treatment, the organisms could be adapted to dispense with glutathione

Starch has been emphasized by several investigators as having a striking effect on the growth of gonococci, but Gould *et al* (106) found that it acts only as a protection against the inhibitory effect of certain samples of agar and can be replaced by charcoal The same authors reported that meat infusion contains an unidentified factor that greatly stimulated the growth of their strains of *N gonorrhoeae*

Factors for hemolytic streptococci Woolley (455), and Sprince and Woolley (392) reported on a factor from liver which promoted the growth of four strains of hemolytic streptococci and one strain of pneumococcus It is insoluble in most organic solvents, adsorbed with difficulty by charcoal but readily by $BaSO_4$, and is stable to acid and alkali The most potent concentrates were about 100 times as active as the starting material, about 10γ per ml of medium gave a maximal effect

Grossowicz (110) has also reported an unknown factor found in tomato juice that is active for hemolytic streptococci It was fairly resistant to heating under neutral or alkaline conditions but was destroyed by peroxide and other oxidizing reagents

Miscellaneous factors Thompson reported (412) that three of nine strains of propionic acid bacteria would not grow in a medium containing the known growth factors but grew satisfactorily when yeast extract was added

All of the indispensable factors for the continued subculture of *Proteus morganii* are known, but Pelczar and Porter (305) found that meat infusion broth contains a stimulatory substance that about doubles the growth in the chemically-defined medium

The same situation exists for *Rhizobium trifolii* according to West and Wilson (437a) Various tissue and microbial extracts contain a heat-stable substance that stimulates growth but is not essential for successful continued transfer of the organism on a synthetic medium

Unidentified factors have been reported for *Bacterium tularense* (407), *Clostridium acetobutylicum* (70) and *Thermobacterium helveticum* (423) but, since not all of the known growth substances were tested, the effects noted may have been due to known rather than to unknown factors

Interchangeability of growth factors

Large quantities of one or more factors may substitute for small quantities of another factor Kögl and van Wagtendonk (160) obtained the same growth of *Staphylococcus pyogenes aureus* with large quantities (5γ per ml) of thiamine and nicotinic acid and no biotin as they did with small quantities (0 05γ) of the two factors and 0 005γ of biotin In other words, increasing the thiamine and nicotinic acid 100-fold dispensed with the need for biotin in the medium Möller (254) reported that a 1000 times larger dosage of *i*-inositol replaced nicotinic acid for *Streptobacterium plantarum*, and in a later paper (256) he stated that large amounts of pure *l*-tyrosine (including the synthetic compound) could meet the thiamine requirements of this organism Snell and Mitchell (376) showed that *Streptococcus lactis* R could be cultured in the absence of unknown

factors (later shown to be folic acid) if thymine was added to the medium Stokes (394) reported that thymine gave complete replacement of folic acid for *S lactis* R, *Streptococcus durans*, three strains of *Streptococcus faecalis*, and partial replacement for *Streptococcus zymogenes* when used in concentrations about 5,000 times that of folic acid Stokstad (399), Stokes (394), and Krueger and Peterson (174) have shown that fair though not optimal growth of *Lactobacillus casei* can be obtained in the absence of the various casei factors if liberal quantities of thymine are contained in the medium Another example of replacement was observed by Snell and Guirard (374) for *S lactis* R, with purified alanine taking the place of pyridoxine About 500γ of alanine were equivalent to 1γ of pyridoxine The authors suggest that alanine may be one of the constituents utilized in the synthesis of pyridoxine Snell and Mitchell (377) found that methionine, adenine, guanine, xanthine, or hypoxanthine could replace *p*-aminobenzoic acid in the nutrition of *Lactobacillus arabinosus* and *Lactobacillus pentosus*[4] Snyder and Broh-Kahn (390) report that cysteine can take the place of hemin for *Hemophilus influenzae* but this report was not confirmed by Bass *et al* (18) The growth of this organism on a hemin-free medium has been a subject of controversy for a long time Ghon and Preyss (103, 104) maintained that such growth was due to small amounts of hematin in the other constituents of the medium Besides the ever-present danger of contaminants, the effect of heat sterilization on the constituents of the medium must not be overlooked Bovarnick (43–46) found that the moderate growth of dysentery bacteria in a medium in which nicotinamide had been replaced by asparagine or glutamic acid was due to the formation of the amide from these two compounds by heat sterilization Many examples of interchangeability among purines and pyrimidines are noted in table 1

Structural specificity of compounds

Many papers dealing with the response of bacteria to large numbers of analogs of the vitamins or parts of their molecules have appeared during the past five years In many cases the analog not only possesses no growth-promoting property but acts as an inhibitor toward the growth factor This inhibition may usually be reversed by addition of larger quantities of the growth factor to the medium The promotion of growth by one compound and inhibition of growth by another is interpreted as indicating that the two compounds play competing roles in some enzyme system As Koser and Saunders (167) listed the papers that dealt with derivatives of thiamine, riboflavin, and nicotinic acid and parts of the molecule of thiamine and pantothenic acid, in general only papers that have been published since the preceding review will be mentioned here

Thiamine Knight (154) found that the pyrimidine and thiazole parts of

[4] In recent personal correspondence Dr Snell says that their medium apparently contained small amounts of *p*-aminobenzoic acid which in the presence of methionine and the purines were adequate for the needs of the bacteria Much larger amounts of *p*-aminobenzoic acid were required for growth when these substances were omitted Landy and Streightoff (195) have also noted that purines markedly increased the sensitivity of *Acetobacter suboxydans* to *p*-aminobenzoic acid

thiamine together were as active on a molar basis as the intact molecule for *Staphylococcus aureus* Sarett and Cheldelin (337), on the other hand, obtained no response to the mixture of pyrimidine and thiazole halves of the thiamine molecule with *Lactobacillus fermentum*

The pyridine analog of thiamine, 2-methyl-4-amino-5-pyrimidyl-methyl-(2-methyl-3-hydroxyethyl)-pyridinium bromide first called "heterovitamin B_1" by Baumgarten and Dornow (22) and Schopfer (345a) but more appropriately named pyrithiamine by Woolley and White (461) has slight growth-promoting properties but is more noteworthy because of its antagonistic action toward thiamine *S aureus* which requires either thiamine or the two parts of thiamine in the medium was rather susceptible to pyrithiamine The inhibition index (ratio of pyrithiamine to thiamine) was 2000 Bacteria that can synthesize thiamine were generally insensitive to pyrithiamine (inhibition index greater than 2,000,000) but the quantity of thiamine synthesized (*e g* 0 02γ/ml in the cells of *E coli*) appeared insufficient to neutralize the quantity of pyrithiamine in the medium (500γ/ml) Wyss (470) tested pyrithiamine as a therapeutic agent against *S aureus* but since non-toxic levels were not antibacterial in the blood, he concluded that it had little chemotherapeutic value Sarett and Cheldelin (338) found pyrithiamine was more inhibitory to the utilization of diphosphothiamine (cocarboxylase) than of thiamine for the growth of *Lactobacillus fermentum*

Riboflavin Derivatives of riboflavin have been investigated by Snell and Strong (381) for potency toward *Lactobacillus casei* and for *Streptobacterium plantarum* and *Bacterium lactis acidi* by Kuhn (175) and Möller (255) Few modifications were equal to the natural compound, and many were inactive The same compounds were tested on rats and the results in general were in good agreement with the bacteriological data Several examples of the competitive action between riboflavin and structurally related compounds, *e g*, 6,7-dichlor-riboflavin (Kuhn *et al*, 179), a phenazine analog (Woolley, 457), propamidine and other antimalarial drugs (Madinaveitia, 235) with respect to lactic acid bacteria and hemolytic streptococci have been reported Kuhn *et al* made the interesting observation that the inhibition ratio of analog to riboflavin increased with time of incubation About six times as much inhibitor was required for a six-day incubation of *S plantarum* as for a two-day period The authors interpreted the results as indicating that the bacteria slowly synthesized riboflavin and eventually overcame the action of the dichlor derivative

Nicotinic acid Pelczar and Porter (301) tested thirteen compounds related to nicotinic acid for activity toward 189 strains of *Proteus vulgaris* and related species Only compounds closely related to nicotinic acid, *e g*, salts, amide, and ester, were active Coramine (diethyl nicotinamide) was found to be active for *P vulgaris* as is also the case for *Streptobacterium plantarum* (Möller and Birkofer, 257), but was found inactive for *Lactobacillus arabinosus* (Teply and Elvehjem, 409) Naturally occurring bound forms of nicotinic acid have been reported and their properties studied by Oser *et al* (297), Andrews *et al.* (8), and Krehl *et al* (171, 172)

Thiazole 5-carboxylic acid (isosteric with nicotinic acid) had about 0 1% of the activity of nicotinic acid for dysentery bacilli (Schmelkes, 345) Pyridine-3-sulfonic acid, the sulfur analog of nicotinic acid, was practically without effect on the growth of *Proteus vulgaris* but strongly inhibited the development of *Staphylococcus aureus* and *Streptobacterium plantarum* (McIlwain, 225, Möller and Birkofer, 257) The inhibition was counteracted by more nicotinic acid or amide and in case of *S plantarum* by heavy metals, especially iron (257)

Picolinic acid also inhibited *S plantarum* Nicotinic acid failed to reverse this inhibition but heavy metals (zinc was the most effective) counteracted the picolinic acid completely (Möller and Birkofer, 258) Wood and Austrian (451) also showed that nicotinamide and cozymase block the action of chemically unrelated compounds Structural similarity appears thus not to be the only basis for antagonism between compounds

The requirement of *Hemophilus parainfluenzae* for cozymase has been shown by Schlenk and Gingrich (343) and Gingrich and Schlenk (104b) to reside in the inability of the organism to link the nicotinamide with the ribose Nicotinamide nucleoside but not other parts of the nucleotide can replace the cozymase molecule

Pantothenic acid Additional reports showing the replacement of pantothenic acid by β-alanine for subspecies of *Corynebacterium diphtheriae* (Evans *et al*, 88, 89), and *Rhizobium trifolii* (West and Wilson, 440) have been published The butyrolactone part of the pantothenic acid molecule is all that is needed by a strain of hemolytic streptococci (Woolley, 453) and by a culture of *Acetobacter suboxydans* (Underkofler *et al*, 417) Either of the two components as well as the pantothenic acid itself stimulates the growth of *Brucella suis* (Koser *et al*, 163) Numerous combinations of analogs of the β-alanine part of pantothenic acid (Weinstock *et al*, 431, Kuhn *et al*, 180, Snell, 366, 367, Barnett and Robinson, 15, McIlwain, 226–233, Pollack, 311, Madinaveitia *et al*, 236) and of the butyrolactone part (Woolley and Hutchings, 458, 459, Subbarow and Rane, 405, Mitchell *et al*, 249) have been tested with various bacteria but the potency of every compound has been either negative or much less than that of the natural component Compounds containing the sulfur analogs of β-alanine, *e g*, pantoyltaurine, are especially interesting since like the sulfa drugs they possess inhibiting properties and their effect can be reversed by pantothenic acid The reversibility suggests that pantothenic acid and pantoyltaurine probably play competing roles in some enzyme system Strains of bacteria that are resistant to pantoyltaurine usually have the ability to synthesize pantothenic acid Resistance is possessed by some strains that occur in nature but can be induced in others

Pyridoxine The specificity of various derivatives of pyridoxine has been determined for *Streptobacterium plantarum* (Möller, 255) and *Lactobacillus casei* (Bohonos *et al*, 38) and comparison made with the response of the rat to the same compounds A good parallelism was found between microbiological and rat potency, but no derivatives were as active as pyridoxine itself

In contrast to the generally lesser activity of analogs of growth factors, Snell

et al (375) have discovered a naturally occurring derivative of pyridoxine, provisionally called pseudopyridoxine, that is several thousand times more active for *Streptococcus lactis* R than the laboratory compound Other lactic acid bacteria showed a similar response to pseudopyridoxine In a later paper, Snell (368) showed that the activity of pyridoxine could be increased many fold by autoclaving the pyridoxine with the medium Autoclaving with amino acids, ammonia, and other constituents of the medium also brought about the conversion As a result of a cooperative effort to solve the problem, Snell (370–372) and Harris *et al* (118) have discovered that the activity of pseudopyridoxine can be accounted for by two synthetic compounds, "pyridoxal" and "pyridoxamine" In these compounds the 4-hydroxymethyl group of pyridoxine is replaced by a formyl (—CHO) or an aminomethyl (—CH_2NH_2) group, respectively These compounds are from 5000 to 9000 times as active as pyridoxine for *S lactis* R, but all three compounds have the same activity for *Saccharomyces carlsbergensis* This difference in response permits a differential assay for pyridoxine and pseudopyridoxine The latter comprises a considerable portion (from less than 5 to more than 50%) of the total vitamin B_6 present in food, body tissues and urine In a personal communication, Dr Snell states that "pyridoxal and pyridoxamine are active for all organisms so far tested which need pyridoxine as well as for some that do not use pyridoxine" It is probable that the functional forms of pyridoxine are compounds identical with or closely related to the aldehyde or amine Some organisms, *e g*, yeast, readily bring about the transformation from the relatively inactive to the very active form The inability of an organism to bring about this transformation might explain failure to grow on a synthetic medium Obviously much work done with pyridoxine should be repeated with the two new compounds Another outgrowth of this work is to reduce the apparent requirement of certain organisms for pyridoxine The quantity of pyridoxine required by many bacteria has always seemed unusually high, but if pyridoxal (or pyridoxamine) is taken as the standard, the amount of these compounds needed places them among the very active growth factors Another oxidation product of pyridoxine, a lactone, has recently been reported by Scott *et al* (348) to promote the growth of *L casei*

p-Aminobenzoic acid (PAB) Since Woods' (452) discovery of the antagonistic action between PAB and sulfanilamide and the consequent identification of PAB as a growth factor, many papers have appeared dealing with various aspects of the relationship between the two compounds The literature on the subject is beyond the scope of this review and only one or two phases of the problem will be mentioned While most papers feature PAB as the antagonist of the sulfa drugs, several other compounds have been found to inhibit their action Methionine, guanine, adenine, hypoxanthine, xanthine, and urethane reverse the action of sulfonamides under some conditions (Bliss and Long, 35, Harris and Kohn, 117, Snell and Mitchell, 377, Johnson, 134a, Kohn and Harris, 161) Combinations of PAB with adenine, hypoxanthine, or xanthine were more effective than would be expected from the amounts of the two compounds used (377) Many sulfur-free compounds (*e g*, 4,4′-diaminobenzophenone, phosphanilic

acid, 4,4′-diaminobenzil, 2,2′-dihydroxybenzil) that are structurally related to PAB and antagonize its growth-promoting action have been prepared and tested by Kuhn and associates (175a, 176, 177) Auhagen (11) reported that on a molar basis *p*-aminobenzoyl-*l*-glutamic acid (PABG) was 8 to 10 times as potent as PAB in reversing the action of sulfanilamide on *Streptobacterium plantarum*, but Williams (443) was not able to confirm this result with a similar organism, *Lactobacillus plantarum* Williams found PAB was 20 times as active as PABG for a closely related organism, *Lactobacillus arabinosus*, and hundreds of times more potent than PABG for *Escherichia coli*, *Clostridium acetobutylicum*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Acetobacter suboxydans*

The existence of other antagonists to the sulfa drugs than PAB has been reported in yeast extract (Loomis *et al*, 205) and in bacterial cultures (Green, 107, Green and Bielschowsky, 108, and Minck, 244) Sevag and Green (351) concluded that the insensitivity of resistant strains of staphylococci is not associated with the formation of PAB However, Landy *et al* (192–194) and Spink *et al* (391) have shown that strains of *Staphylococcus aureus* which are resistant to sulfonamide drugs produced on the average from 40 to 70 times as much PAB as non-resistant strains of the same organism The exclusion of PAB on the basis of unlike chemical properties and quantitative data does not appear to rest on too firm a basis because of the uncertainty regarding the properties and quantitative determination of bound PAB

Rubbo *et al* (335) reported that *p*-aminophenylacetic acid was ten times as active as PAB for *Clostridium acetobutylicum*, but Wyss *et al* (470a) and Lampen and Peterson (183) obtained an activity of only about 0 1% of that given by PAB When tested on *Acetobacter suboxydans*, which requires PAB, *p*-aminophenylacetic acid showed 2 per cent of the activity of PAB Many other compounds related to PAB have been tested by the above groups of workers but most of these had little activity

Biotin Two forms of biotin have been reported to exist in nature and many papers dealing with their isolation, structure and potency have appeared (Kögl and associates, 157–159, du Vigneaud and associates, 419, 421) The first obtained in crystalline form from egg yolk by Kögl and Tönnis (158), is designated α-biotin by Kögl and ten Ham (157) and the second, obtained by du Vigneaud and associates (421) from liver is called β-biotin by the Dutch investigators In a recent review on biotin, Melville (240a) points out that the structure proposed for α-biotin has not been confirmed by synthesis as has been done with β-biotin and also notes that the conclusion of Kögl and associates regarding the structure of β-biotin is not in agreement with the work of the American investigators The existence of two forms of biotin therefore appears to be an open question Aside from differences in structure, a difference in activity is said to distinguish the two compounds α-Biotin is reported to be only one-half as active for yeast as β-biotin No reports comparing the activity of the two biotins for bacteria have been noted

Various compounds related to β-biotin have been tested for their bacterial activity The methyl ester is as active as the free acid for some bacteria, *e g*,

Staphylococcus aureus, *Clostridium butylicum*, but by others, *e g*, *Lactobacillus casei*, *Streptobacterium plantarum*, it is used with difficulty (Möller, 255, Stokes and Gunness, 395, and Tomlinson and Peterson, 416) Synthetic biotin had the same potency as natural biotin, *dl*-biotin was only 50% as potent, and *l*-biotin and *dl*-allobiotin had only slight activity (due perhaps to contamination with the active *d*-biotin) for *L casei* and *L arabinosus* (Stokes and Gunness, 396a)

Several papers (Dittmer *et al*, 74, Dittmer and du Vigneaud, 75, Lilly and Leonian, 203, and Stokes and Gunness, 396a) deal with the activity of compounds structurally related to biotin None of these compounds have any real growth-promoting effect but several of them have antibiotin activity, and the inhibition can be reversed by the addition of more biotin Most of the work has been done with desthiobiotin (a sulfur-free compound) and *L casei* Biotin sulfone, however, has greater antibiotin activity than desthiobiotin. Some bacteria that require biotin (*L arabinosus* and *Rhizobium trifolii*) were not inhibited by large additions of desthiobiotin to the medium In contrast to yeasts, which are able to reform the sulfur ring, bacteria do not seem to have this synthetic ability Benzimidazole, which is also structurally related to biotin, inhibited the growth of *Streptococcus lactis* R and *Escherichia coli* Biotin did not counteract the effect of benzimidazole on either organism but guanine and adenine overcame its action on *E coli*, and uracil removed the inhibition toward *S lactis* R (Woolley, 456)

By treatment of urine or vitab hydrolysates, Burk and Winzler (47) have obtained biotin-like products that have been designated miotin, tiotin, and rhiotin, depending on their reaction to heat, avidin, yeast, and rhizobium bacteria As these products have not been isolated and identified, and as many of the properties attributed to them are possessed by known derivatives of biotin, *e g* desthiobiotin and diaminocarboxylic acid, little advantage seems to be gained from the introduction of these terms

Pimelic acid, discovered by Mueller (263, 264) to be a growth factor for the diphtheria bacillus, is apparently a precursor of biotin (du Vigneaud *et al*, 420) No other biotin-requiring bacterium has been found able to use pimelic acid in place of biotin Wright (462) and Hutner (129) obtained negative results with *Lactobacillus casei* and *Rhodospirillum rubrum*, respectively

SYNTHESIS OF GROWTH FACTORS

In table 1, a record indicated by the letter S was made of the synthesis of growth factors which need not be supplied to the bacteria named Besides these, there is a larger number of bacteria that need no preformed growth factors Bacteria that can grow on a sugar + salts medium must of course synthesize all the growth factors required in their metabolic processes A list of all bacteria reported to synthesize one or more growth factors is given in table 3 Probably less than ten species have been tested for their ability to synthesize all of the B vitamins listed in table 1, and less than a dozen have been tested for the presence or absence of as many as four of these compounds On the other hand, it is generally assumed that all of these compounds are utilized in the growth and

metabolism of all bacteria The limited information available lends support to this view The most complete data bearing on the subject are those of Thompson (411) He reports data for eight B-vitamins in cells and medium of bacteria from five different genera grown for 24 hours on the same medium Arranged

TABLE 3

Synthesis of growth factors by bacteria

FACTOR	ORGANISMS REFERENCES, AND CONCENTRATION RANGE
p-Aminobenzoic acid	*Alcaligenes faecalis* (193), *Aerobacter aerogenes* (193), *Bacillus megatherium* (193), *B subtilis* (193), *B vulgatus* (193), *Brucella abortus* (193), *Clostridium botulinum* (193), *C sporogenes* (193), *C tetani* (193), **Corynebacterium diphtheriae* (193), *Diplococcus pneumoniae* (193), *Eberthella typhosa* (193), *Escherichia coli* (193), *Klebsiella pneumoniae* (193), *Lactobacillus casei* (193), *L delbrückii* (193), **Myocbacterium tuberculosis* (193), *M stercoris* (193), **M smegma* (193), *Neisseria gonorrhoeae* (191), *Proteus vulgaris* (193), **Pseudomonas aeruginosa* (193), *Salmonella paratyphi* (193), **S schottmuelleri* (193), *Serratia marcescens* (193), *Shigella dysenteriae* (193), *S paradysenteriae* (193), **Staphylococcus albus* (193), **S aureus* (193), staphylococci (resistant strains (391)), *Streptococcus hemolyticus* (193), *S salivarius* (193), **S scarlatinae* (193) Range 0 003γ/ml, cells and culture filtrate, *C sporogenes*, to 3 3γ/ml, *S aureus*
Biotin	*Alcaligenes faecalis* (48, 187), *Acetobacter suboxydans* (417), **Aerobacter aerogenes* (187, 411), *Azotobacter vinelandii* (200), *Bacillus anthracis* (187), *B subtilis* (187), *B vulgatus* (48), *Bacterium aerogenes* (48), *Clostridium acidi urici* (14), *Eberthella typhi* (187), **Escherichia coli* (48, 101, 187, 241), *Klebsiella pneumoniae* (187), **Mycobacterium tuberculosis* (187), **Phytomonas tumefaciens* (234), *Proteus vulgaris* (48, 411), *Pseudomonas aeruginosa* (187), **P fluorescens* (411), *Sarcina lutea* (187), *Serratia marcescens* (187, 411), **Staphylococcus aureus* (187), *Thiobacillus thiooxidans* (290) Range 0 0005γ/ml, cells and culture filtrate, *A faecalis*, to 0 035 γ/ml, *P tumefaciens*
Nicotinic acid	*Alcaligenes faecalis* (48), *Aerobacter aerogenes* (411), **Azotobacter vinelandii* (200), *Bacillus mesentericus* (48), *B vulgatus* (48), *Bacterium aerogenes* (48), **Clostridium butylicum* (411), *Corynebacterium diphtheriae* (424), *Escherichia coli* (48), **Proteus vulgaris* (411), *Pseudomonas fluorescens* (411), *Serratia marcescens* (411), *Shigella paradysenteriae* (169), *Thiobacillus thiooxidans* (290) Range 0 028 γ/ml, cells and culture filtrate, *E coli*, to 4 6 γ/ml, *A vinelandii*

* Denotes organisms that have been reported to be good producers of the factor, *i e*, approximately one half or more of that reported for the best producer

TABLE 3—*Continued*

FACTOR	ORGANISMS REFERENCES, AND CONCENTRATION RANGE
Pantothenic acid	**Aerobacter aerogenes* (411), **Azotobacter vinelandii* (200), **Clostridium butylicum* (411), *Phytomonas tumefaciens* (234), *Proteus vulgaris* (411), *Pseudomonas fluorescens* (411), *Rhizobium meliloti* (217), *Serratia marcescens* (411), *Thiobacillus thiooxidans* (290) Range 0 0303 γ/ml, cells and culture filtrate, *P vulgaris*, to 0 99 γ/ml, *A vinelandii*
Pyridoxine	*Aerobacter aerogenes* (411), **Clostridium butylicum* (411), *Lactobacillus arabinosus* (38), *L pentosus* (38), *Leuconostoc mesenteroides* (38), *Proteus vulgaris* (411), **Pseudomonas fluorescens* (411), **Serratia marcescens* (411), *Thiobacillus thiooxidans* (290) Range 0 0049 γ/ml, cells and culture filtrate, *P vulgaris*, to 0 0242 γ/ml, *P fluorescens*
Riboflavin	*Alcaligenes bookeri* (415), *A faecalis* (48), *A viscosus* (330), **Acetobacter suboxydans* (330, 417), *Achromobacter delicatulum* (415), *A radiobacter* (330), *Aerobacillus polymyxa* (330), *Aerobacter aerogenes* (330, 411, 415), *A cloacae* (415), *A oxytocum* (415), *Azotobacter agile* (393a), *A chroococcum* (330, 393a), *A vinelandii* (200, 330, 393a), *Bacillus alboluctis* (415), *B cereus* (415), *B cohaerens* (415), *B graveolens* (415), *B globigii* (330), *B mesentericus* (48), *B mycoides* (330, 415), *B niger* (330, 415), *B rotans* (415), *B ruminatus* (415), *B subtilis* (330, 415), *B vulgatus* (48, 415), *Bacterium aerogenes* (48), *B brassicae* (381), *B herbicola*, (330), **Clostridium acetobutylicum* (330, 471), *C acidi urici* (14), **C butylicum* (411), **C butyricum* (425a), *C felsineum* (330), *C pasteurianum* (425a), *C roseum* (330), *Corynebacterium diphtheriae* (67, 73, 88, 330, 424), *Eberthella typhi* (330), *Escherichia coli* (48, 330, 415, 461), *Flavobacterium sulfuricum* (330), *Klebsiella pneumoniae* (415), *Lactobacillus arabinosus* (330), *L brassicae* (381), **L delbrückii* (425a), *L helveticus* (2), *L pentosus* (381), *Leuconostoc mesenteroides* (381), *Micrococcus casei* (415), *M cereus* (415), *M citreus* (415), *M freudenreichii* (415), *M percitreus* (415), *M perflavus* (415), *M subflavus* (415), *M ureae* (415), **Mycobacterium tuberculosis* (40, 333, 401), *M smegmatis* (330), *Neisseria catarrhalis* (330), **Phytomonas tumefaciens* (234, 330), *Proteus vulgaris* (48, 330, 411, 415), *Pseudomonas aeruginosa* (330), *P fluorescens* (292, 294, 330, 411), *P pyocyaneus* (292, 294), *Rhizobium trifolii* (437), *Salmonella schottmuelleri* (330), *Sarcina lutea* (415), *Serratia marcescens* (330, 411, 415), *Shigella dysenteriae* (330), *Shigella paradysenteriae* (80), *Spirillum serpens* (330), *Staphylococcus albus* (330, 415), *S aureus* (289, 330, 415), *S flavus* (289), *Streptococcus bovis* (415), *S lactis* (381), *Thiobacillus thiooxidans* (290) Range 0 02 γ/ml, *Bacterium herbicola*, to 8 5 γ/ml, *Acetobacter suboxydans*

TABLE 3—*Concluded*

FACTOR	ORGANISMS, REFERENCES, AND CONCENTRATION RANGE
Thiamine	**Alcaligenes faecalis* (48), *Aerobacter aerogenes* (411), *Azotobacter vinelandii* (200), *Bacillus mesentericus* (48), **B vulgatus* (48, 111), **Bacterium aerogenes* (48), *Clostridium butylicum* (411, 461), *Corynebacterium diphtheriae* (88, 424), *Escherichia coli* (48, 102, 461), hemolytic streptococci (286, 461), *Lactobacillus arabinosus* (461), *L casei* (461), *Propionibacterium freudenreichii* (408), *P pentosaceum* (361), **Proteus vulgaris* (48, 411), *Pseudomonas fluorescens* (411), *Rhizobium trifolii* (437), *Serratia marcescens* (411), *Streptococcus durans* (286), *S faecalis* (286), *S liquefaciens* (286), *S zymogenes* (286), *Thiobacillus thiooxidans* (290) Range 0 0032 γ/ml, cells and culture filtrate, hemolytic streptococci, to 0 150 γ/ml, *B vulgatus*
Vitamin K	*Bacillus coli* (68, 69, 296), *B cereus* (4), *B mycoides* (4), *B subtilis* (4), *Bacterium aerogenes* (4), *B bifidum* (296), *B flexneri* (4), *B proteus* (4), *B typhosum* (4), *Erythrobacillus prodigiosus* (4), *Escherichia coli* (4), *Microbacterium lacticum* (296), *Mycobacterium phlei* (460), *M tuberculosis* (4, 6a), *Sarcina lutea* (4), *Staphylococcus aureus* (4), *Streptococcus, faecium* (69) Range Expressed as 2 methyl-1,4-naphthoquinone From 8 γ per gram dry weight of cells, *B bifidum*, to 152 γ per gram dry weight of cells, *B subtilis*
Norite eluate factor, folic acid, vitamin B_c	*Aerobacter aerogenes* (465), *Azotobacter vinelandii* (200), *Bacillus lactis acidi*, *B brassicae* (124), *Clostridium acidi urici* (14), *C butylicum* (411), *Escherichia coli* (241), *Lactobacillus arabinosus* (124), *L gayonii* (124), *L pentosus* (124), *Leuconostoc mesenteroides* (124), *Proteus vulgaris* (411), *Pseudomonas fluorescens* (411), *Serratia marcescens* (411) Range Not expressed in terms of a pure compound
Miscellaneous	
Anti-hemorrhagic compounds	*Mycobacterium phlei* (460)
Inositol	*Aerobacter aerogenes* (411), *Clostridium butylicum* (411), *Proteus vulgaris* (411), *Pseudomonas fluorescens* (411), *Serratia marcescens* (411)
Uracil	*Bacterium typhosum* (329), *Staphylococcus aureus* (329)
Vitamins B_{10} and B_{11}	*Mycobacterium tuberculosis* (243)
P factor	*Brucella abortus* (107)

roughly in descending order of growth-factor productivity (cells and medium) the bacteria were as follows *Pseudomonas fluorescens*, *Aerobacter aerogenes*, *Serratia marcescens*, *Clostridium butylicum*, *Proteus vulgaris* In the cells the compound present in smallest amount, biotin, varied from 1 7 (*P vulgaris*) to 7 1 (*P fluorescens*) micrograms per gram of dry cells (equivalent to from 2300

to 4000 ml. of medium). The compound present in next to largest amount, nicotinic acid, ranged from 200 to 300γ. Inositol, which has not been reported to be required by any bacteria, amounted to from 870 to 1700γ.

The distribution of the compounds between cells and cell-free medium was generally in favor of the medium. This was outstandingly so for biotin, where about 90% of the total was found in the medium and only 10% in the cells. Thiamine was distributed about equally between cells and medium. Since the incubation period was only 24 hours, Thompson concluded that the compounds found in the medium represented secretion rather than autolysis of dead cells. Confirming evidence was obtained for biotin in case of *P. vulgaris* by successive analyses during the incubation period. Based on the percentage of the total produced in 48 hours, the biotin in the medium "led" that in the cells.

The effect of adding each growth factor in turn to the medium on the synthesis of the others by *A. aerogenes* was tested with negative results. Likewise the addition of about 1 microgram of riboflavin per ml. of medium caused no increase in the riboflavin content of the cells and even slightly more riboflavin was synthesized than when no riboflavin was added. One might say that the cells "preferred" to synthesize their riboflavin rather than to take it from the medium.

Burkholder and McVeigh (48) reported data for the synthesis of four growth factors by six species of intestinal bacteria, grown for 48 hours. Two of the species, *A. aerogenes* and *P. vulgaris*, were the same as those Thompson used. Calculated to a dry matter basis (assumed to be 25%), Burkholder and McVeigh's figures are in general higher than Thompson's, in case of thiamine, 16 to 18 times higher. These differences are not to be stressed, since variation in strain, medium, aeration, incubation period, etc. probably account for them. By selection of strains and modifying the cultural conditions, the productivity can certainly be increased many fold.

Sevag and Green (350) have shown that tryptophane is required for the synthesis of arylamines (PAB ?) by *Staphylococcus aureus*. Lysine, threonine, and alanine appear to be required for the synthesis of pyridoxine by *Lactobacillus arabinosus* (Stokes and Gunness, 396).

Besides the papers dealing with the synthesis of vitamins by pure cultures of bacteria, there is a considerable literature that is concerned with the synthesis of B vitamins by bacteria in the intestinal tract of animals and man. The synthesis of B vitamins in the rumen of cattle is sufficient to meet all the needs of the animal for these vitamins. In the rat, a biotin deficiency cannot be obtained without the use of egg white to bind the biotin that is produced by bacteria in the cecum and lower intestine. The synthesis of the B vitamins in the intestinal tract of animals thus becomes a matter of considerable nutritional importance. Likewise this synthesis is a complicating factor in the interpretation of the data obtained in feeding experiments. A number of papers dealing with the effect of diet, species, and other factors on the intestinal synthesis of growth factors have appeared for the following compounds: biotin (222, 246, 278, 427), nicotinic acid (246, 427), pantothenic acid (221, 246, 427), pyridoxine (221, 246, 262, 427), riboflavin (221, 236a, 246, 262, 427), thiamine (1, 221, 246, 427), inositol (246, 455a), vitamin K (221), and folic acid (246).

BACTERIOLOGICAL ASSAYS FOR GROWTH FACTORS

One of the striking developments in the field of growth factors in recent years is the use of microörganisms for the quantitative determination of these compounds in foods, normal and abnormal tissues, blood, feces, urine, bacteriological media, vitamin products, and many other biological materials Probably more analyses are made by microbiological than by chemical methods Many of the B vitamins cannot yet be determined by chemical methods and the advent of microbiological procedures has been of enormous advantage to the industries that manufacture products featuring vitamin content Of the ten vitamins in the B-group, eight can be readily determined by means of bacteria, four can be assayed by means of yeasts, and four lend themselves well to chemical determination Inositol can be determined by means of yeast but not with bacteria, because as yet no bacterium has been shown to require this compound in the culture medium

While the use of bacteria for assay purposes had been suggested previously, the first widely used method was that proposed by Snell and Strong in 1939 (382) for riboflavin by means of *L casei* (A T C C 7469) In the next five years nearly a score of papers dealing with this one method were published During the same period this bacterium has been used successfully for the determination of pantothenic acid, biotin, and *L casei* factor (vitamin B_c, folic acid) and less satisfactorily for nicotinic acid and pyridoxine Its growth is only slightly stimulated by thiamine and *p*-aminobenzoic acid, hence it is unsuitable for the determination of these compounds Nearly forty papers have been published dealing with the use of this one microörganism This is approximately one-half of all papers published on bacteriological assay methods in the past five years

Many of the methods that have been published have been found to have serious faults which came to light as the methods were applied to a wide variety of materials Sensitivity to compounds (*e g*, fatty acids) never suspected of playing a part in the nutrition of bacteria, free and bound forms of vitamins occurring in nature, effect of structure on activity (leading to a rational explanation of the action of certain drugs) are some of the products of research on quantitative methods

Theoretically an assay method should be possible for any compound that is required by a microörganism, but in practice some microörganisms are more satisfactory than others Some of the necessary conditions that should be fulfilled are as follows

1 The medium should contain all constituents that are necessary for optimum development and activity of the microörganism other than the factor to be determined Such a medium is indicated by the production of a low turbidity or acidity in the absence of the factor and optimum growth and formation of products in its presence With an excess of the factor the development of the microörganism should be equal to that in a natural medium of comparable sugar and nitrogen content

2 The response of the microörganism (as measured by cell growth, products,

or other index) to increasing quantities of the factor should be regular and preferably proportional Equal response to all forms of the compound on a molar basis is desirable A striking example of non-equivalence is illustrated by the difference in response of *L casei* to pyridoxine, pyridoxal, and pyridoxamine *No satisfactory bacterial assay for all three of these compounds appears to be available at the present time*

Measurement of combined forms of growth factors is particularly difficult While some of these, *e g*, coenzyme I and riboflavin nucleotides, appear to be equivalent on a molar basis to the free compound, in other cases (*e g* biotin, pantothenic acid), the bound form is unavailable to the assay organism The growth factor must then be set free by means of chemical reagents or enzymes Some growth factors are labile to acid and alkali, and enzymes do not always release the compound from its bound form The ideal microörganism might be one that has strong amylolytic and proteolytic powers so as to enable it to use all forms of the compound and thus approximate the action of digestive enzymes in animal nutrition On the other hand, the microörganism should not respond to degradation products of the compound Up to the present no bacterium possessing all these desirable features has been found

3 In the assay of natural materials, the responses at different levels should give the same value when this is calculated per gram or other unit of the material Irregular values are indicative of another factor or factors in the material which may be stimulatory or inhibitory at different levels

4 Repeated assays of the same material should check, *e g*, within 5%. A suitable sample repeatedly analyzed serves as a reference material and should be included in every large series of analyses as a useful check on the assay as a whole

5 The sample should contain no inhibitory or toxic substances If such substances are present, the results will probably be variable, as mentioned under 3 Such a substance may be present in the original material or be produced by the treatment of the material, *e g*, decomposition products or excess salts

6 A standard inoculum should be used To start with a sturdy and stable microörganism is essential The stock culture should be carried on a medium that maintains the organism in a stable condition, and the inoculum should be developed in the same way each time Obviously such points as age and size of inoculum are of great importance

7 The microörganism should preferably be non-pathogenic If the method is to be widely used and consequently by technicians, a pathogen is dangerous, and necessary precautions reduce the number of assays that can be performed in a given time

8 The method should be rapid In research work, the time required for an assay should be 1 to 3 days, and for control work in industry 5 to 20 hours

Even if these requirements are met to a reasonable degree, there still is no substitute for skill and experience on the part of the analyst Perfection in the method should not be expected Even after more than half a century of use and scores of modifications the Kjeldahl method for total nitrogen is still not perfect

TABLE 4

Bacteriological assay methods

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
p-Amino-benzoic acid	*Acetobacter suboxydans*	Turbidity	48	0–10 mγ	189, 195, 247
	Clostridium acetobutylicum	Turbidity	20–24	0–0 15 mγ	184
	Lactobacillus arabinosus	Acidity	72	0–0 05 mγ	201
Biotin	*Clostridium butylicum*	Turbidity	72	0–0 1 mγ	181
	Lactobacillus arabinosus	Acidity	72	0–0 25 mγ	466
	Lactobacillus casei	Acidity	72	0–1 mγ	74, 188, 353, 354, 395, 416, 445
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–1 mγ	100
	Rhizobium trifolii	Turbidity	72	Yeast extract	440
Choline	*Pneumococcus* Type III	Turbidity	12–24	0–6 γ	12
Coenzymes I and II	*Hemophilus influenzae*	Nitrite production	48	0–0 037 γ	121
	H parainfluenzae	Turbidity	24–30	Yeast extract = 0–0 18 mg of fresh yeast	160a
	H parainfluenzae	Turbidity	40–42	2 5–20 mγ	310
	H parainfluenzae	Turbidity	24–29	0–0 06 γ	261
Nicotinamide	*Shigella dysenteriae*	Turbidity	16–22	0–0 025 γ	132
Nicotinic acid	*Acetobacter suboxydans*	Turbidity	48	0.25–3 0 γ	138
	Bacillus proteus	Turbidity	30	0–0 1 γ	214
	Lactobacillus arabinosus	Acidity	72	0–0 1 γ	8, 60, 109, 131, 173, 239, 387 388
	L arabinosus	CO_2 liberation by acid formed	3	0–13 3 mγ	10
	L casei	Acidity	72	0–0 1 γ	188
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–0 1 γ	100

TABLE 4—*Continued*

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
Nicotinic acid (*Cont'd*)	*Dysentery bacillus* (*Shigella?*)	Acidity	4 days	001–010 γ	79
Pantothenic acid	*Lactobacillus arabinosus*	Acidity	72	0–0 02 γ	362
	L casei	Acidity	72	0–0 20 γ (Ca salt)	20, 50, 188, 202, 275, 276, 307 308, 355, 393, 398, 403, 425, 442, 463
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–10 mγ	100
	Proteus morganii	Turbidity, pH or bacterial nitrogen	24	0–1 mγ	303, 304
(β-Alanine)	*Corynebacterium diphtheriae*	Bacterial nitrogen	70	0–1.5 γ	342
Pyridoxine* (pyridoxal, etc)	*Lactobacillus casei*	Acidity	72	0–0 1 γ	51a, 188, 369
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–0.25 γ	100
"Pseudopyridoxine"	*Streptococcus lactis* R	Turbidity	16	0–0 3 γ	134, 368, 375
Riboflavin	*Lactobacillus casei*	Acidity	72	0–0 05 γ	7, 16, 17, 20, 56, 87, 133, 140, 188, 318, 319, 347, 382, 402, 404, 406, 428, 429
	L casei	Acidity	72	0–20 mγ	206
(small amounts)	*L jugurt*	Acidity	72	0–0 1 γ	51
Thiamine	*Lactobacillus fermentum*	Turbidity	16–18	0–5 mγ	337
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–3 mγ	100
	Propionibacterium pentosaceum	CO_2 evolution	4	0–0.25 γ	356, 361
	Staphylococcus aureus	Turbidity	36	0–0.5 mγ	436
	Streptococcus salivarius	Turbidity	24	0–0.2 mγ	283
Norite eluate factor	*Lactobacillus casei*	Acidity	48	0–300 γ solubilized liver fraction	379

TABLE 4—*Concluded*

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
Folic acid	*Streptococcus lactis* R	Turbidity	24	0–200 γ liver extract B	207, 248
	Lactobacillus casei	Acidity	72	0–0 003 γ folic acid concentrate	188
Vitamin B_6	*L casei*	Acidity	30–72	0–1 mγ	174, 410
	S lactis R	Acidity	30–72	0–8 mγ	410
	S lactis R	Turbidity	16	0–5 mγ	174

* Pyridoxine is inactive for *L casei* and other lactic acid bacteria, whereas pyridoxal and pyridoxamine are very active All these terms are retained in this review since no single compound has as yet been agreed on by investigators to express the vitamin B_6 activity of natural materials

If used with skill and judgment, microbiological assays can be valuable and useful tools

In table 4 are listed the principal bacteriological methods that have been proposed for the quantitative determination of various growth factors In compiling this table only those methods are included that have been definitely proposed for the purpose and their application to natural materials worked out to some degree

Next to *L casei*, the organism most used for assays is *L arabinosus* It is employed almost exclusively for estimating nicotinic acid, and occasionally for biotin and *p*-aminobenzoic acid Since it also requires pantothenic acid (table 1), presumably it could be used for the assay of this compound *Leuconostoc mesenteroides* is a bacterium that requires practically the same growth factors as *L casei*, and recently Gaines and Stahly (99, 100) have suggested its use for five different assays, but the methods have not yet been applied to biological materials It is only in practice that the reliability and limitations of a method can be ascertained *L mesenteroides* has one advantage over *L casei* in that it can be used for thiamine assay, but it also has a disadvantage in not being suitable for riboflavin because it synthesizes this compound Its response to the new *L casei* and *S lactis* factors and its sensitivity to higher fatty acids have not been determined

A single organism and a standard medium suitable for as many assays as possible would be highly desirable in control work, and Landy and Dicken (188) proposed such a medium adaptable for the determination of six growth factors by means of *L casei* This medium, however, did not contain all of the factors required by *L casei* in optimal amounts It can also be much improved by the inclusion of the pure form of newly isolated growth factors, and the addition of stimulatory substances (Sprince and Woolley, 392, Teply and Elvehjem, 410) The most serious known disadvantage to the use of *L casei* is its sensitivity to

higher fatty acids To date no completely satisfactory procedure has been developed for the removal of these interfering substances Unfortunately most lactic acid bacteria seem to be sensitive to small amounts of higher fatty acids but a systematic study of the lactic acid bacteria might reveal a more suitable organism than *L casei*

A glance at table 4 shows that turbidity and acidity are the criteria most widely used for measuring the response of the organism Nitrite production and synthesis of cellular nitrogen have been used in a few cases

One of the features most strongly favoring bacteriological methods is the small quantity of the compound required for a determination This ranges from 10^{-7} to 10^{-3} microgram of biotin to 0 05 to 0 5 microgram of nicotinic acid—quantities that are far beyond the reach of most chemical methods

DESTRUCTION OF GROWTH FACTORS

For some time bacteria have been known to destroy ascorbic acid in the medium and in the intestinal tract of cattle Foster (95) describes a new species of bacteria, *Pseudomonas riboflavinus*, that oxidizes riboflavin to lumichrome stoichiometrically according to the equation

$$\underset{\text{riboflavin}}{C_{17}H_{20}O_6N_4} + 5\tfrac{1}{2}O_2 \longrightarrow \underset{\text{lumichrome}}{C_{12}H_{10}O_2N_4} + 5CO_2 + 5H_2O$$

Mirick (244) isolated a soil organism which, on the basis of oxygen uptake, apparently oxidized *p*-aminobenzoic acid to carbon dioxide, water, and ammonia By culturing the organism in the presence of anthranilic (*o*-aminobenzoic) acid, the cells became adapted to the destruction of this compound In a medium containing nicotinic acid as the sole source of carbon *Pseudomonas fluorescens* and *Serratia marcescens* destroyed the compound In a medium devoid of nicotinic acid both organisms synthesized it (Koser and Baird, 161a)

FUNCTION OF GROWTH FACTORS

The role of certain growth factors, *e g*, riboflavin and thiamine, as constituent parts of coenzymes has been mentioned so many times that it need not be reviewed here By analogy it is generally assumed that the other growth factors serve in a similar capacity However, most of the recent data in support of this view are general rather than specific in character Kligler *et al* (148, 149) reported that nicotinic acid and thiamine are required if glucose is present in the medium, but not if it is absent Many papers have been published showing that thiamine (119, 321–323, 358, 360, 365), riboflavin (2, 143), pantothenic acid (26, 78, 120), and nicotinic acid (or coenzyme I) (82, 211) increase respiration (oxygen uptake, carbon dioxide production or methylene blue reduction) by cells (*e g*, *Staphylococcus aureus*, *Propionibacterium pentosaceum*, *Proteus morganii*, *Lactobacillus mannitopoeus*, dysentery bacilli, *Hemophilus parainfluenzae*) acting on various substrates (*e g*, glucose, lactate, pyruvate)

A more specific type of function for a growth factor in catabolic processes has been uncovered by Gunsalus and coworkers (23, 24, 112–115) in relation to the

decarboxylation of tyrosine by strains of *Streptococcus faecalis* In a series of papers, these workers have shown first, a marked apparent requirement for pyridoxine in the decarboxylation process, second, that pseudopyridoxine is more active than pyridoxine, third, that pyridoxal possesses this increased activity, and fourth, that adenosine triphosphate (ATP) functions with pyridoxal in the decarboxylation process A synthetic compound, presumably phosphorylated pyridoxal, was prepared and found to function with an enzyme preparation from *S faecalis* The exact structure of the compound is still to be determined

The above results do not correlate entirely with the work of two English investigators (Gale and Epps, 97a, 98) on the coenzyme involved in the decarboxylation of tyrosine and lysine by *E coli* and *S faecalis* These authors report that pyridoxine had no coenzyme activity In the second paper, they report purification of the coenzyme about 15,000 times and state that their preparation contained no phosphorus It did contain C, H, and N but the percentage content was not that of pyridoxine However, they did not test pyridoxal for coenzyme activity nor did they determine the pyridoxine (or pyridoxal) content of their coenzyme preparation The discrepancies between the results obtained by the two groups of workers will probably be cleared up shortly Another function for pyridoxal is reported by Schlenk and Snell (344) and by Snell (373) who showed that it is involved in transamination reactions

In synthetic processes, Sevag and Green (349) showed that pantothenic acid was required for the building of tryptophane by certain strains of *Staphylococcus aureus*, and Stokes and Gunness (396) found that pyridoxamine (or pyridoxal) was needed in the synthesis of lysine, threonine, and alanine by *Lactobacillus arabinosus, Lactobacillus casei,* and *Lactobacillus delbrückii* Stokes (394) explains the interchangeability of thymine and folic acid in the nutrition of various lactic acid bacteria as evidence for the view that the role of folic acid is to function in the synthesis of thymine This in turn is needed for the building of nucleic acids *p*-Aminobenzoic acid is not required for the growth of the tubercle bacillus but in high concentrations promotes the formation of an unidentified yellow pigment (Mayer, 238)

Meyer (240) reported that biotin increases enormously the activity of the mucolytic enzyme, lysozyme The increased lysis, of both living and acetone-dried cells of *Micrococcus lysodeikticus* when 10γ of biotin was added to the substrate, ranged from 8 to 250 times A note by Laurence (199) reported that lysozyme binds biotin and that the avidin-biotin complex has lysozyme activity

However, such a relationship of lysozyme to biotin and avidin does not seem to hold as judged by other work Alderton *et al* (3) reported that crystalline lysozyme contained little or no biotin or avidin Additions of biotin to pure or impure lysozyme preparations did not enhance the lytic activity of the enzyme Conversely, avidin preparations were essentially free of lysozyme Previously Woolley and Longsworth (459a) had found that highly purified avidin was devoid of lysozyme activity

Two papers (Miller *et al*, 242, Williams *et al*, 444) attempting to link growth factors with a number of highly purified enzyme proteins have given negative

results About 15 of the common enzyme proteins, many of which had been prepared in a crystalline state, were analyzed for seven of the well-known growth factors, *e g* biotin, etc Although small quantities of these compounds were found in the enzyme proteins, the amounts were too small to be considered constituent parts of the molecule except perhaps for inositol in amylase and thiamine in carboxylase Assuming only a single mole of the factor in one molecule of protein, a molecular weight far greater than that ascribed to these proteins would be required

While there are more data associating growth factors with breakdown than with synthetic processes, considerable information is being accumulated linking growth factors with the building of the cell as well as with its maintenance The term growth factor will appear more appropriate as more information is obtained linking it with constructive processes

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YEAST GENETICS[1]

Life Cycles, Cytology, Hybridization, Vitamin Synthesis, and Adaptive Enzymes

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CONTENTS

I LIFE CYCLES

Common bakers' and brewers' yeast, *Saccharomyces cerevisiae*, exists in both haplophase and diplophase There are two mating types in the haplophase, and haploid cells of opposite mating type copulate to produce diploid cells Winge (76) in Copenhagen, and Kruis and Šatava (26) in Prague, showed that the standard vegetative cells of *S cerevisiae* are diploid, produced by copulation of two spores or gametes derived from spores The diploid nuclei undergo reduction at spore formation to produce four haploid ascospores The large, ellipsoidal vegetative yeast cell is produced by the fusion of two round haploid gametes derived from ascospores Winge established the facts of this life cycle beyond question by a classical series of observations on the germination of ascospores and fusion of haploid cells Winge and Laustsen (78–82) in a series of notable papers, showed that colonial characteristics, fermentative ability, and cell shape are under the control of genes which segregate at the reduction division

The Distribution of Haplophase and Diplophase

Winge was the first to distinguish clearly between haplophase and diplophase yeast cultures, and we have corroborated his observations with some slight modifications Workers familiar with other biological material may question the propriety of speaking definitely of haplophase and diplophase in organisms where the cytological facts have not been conclusively demonstrated I shall therefore summarize all the arguments, Winge's reinforced by ours, for distinguishing haplophase and diplophase I should preface these rules by saying

[1] This work was supported by a grant from Anheuser-Busch, Inc , St Louis

that over four-fifths of the cultures which one encounters are easily characterized by microscopic examination. They are either obviously haploid or diploid, as shown simply by size, shape, and aggregation of the cells. The reasons for classifying them are as follows:

a. The large vegetative cells, which we call "legitimate diploids" produce viable four-spored asci. These spores germinate to produce smaller, usually round, cells, which we call "haploid." The latter multiply vegetatively, generally maintaining their specific cell-shape and size.

b. Two of these smaller cells may fuse to produce a large "diploid" cell capable of vegetative multiplication. While the large cell is undergoing vegetative reproduction, it retains its characteristic ellipsoidal shape and size. Under certain conditions, this diploid cell can be induced to sporulate. Spores from it in turn produce haploids and the process can be repeated indefinitely.

c. The large cells which we recognize as diploids are extraordinarily stable in their genetic characeristics when they are grown under conditions in which sporulation does not occur. Transferring the cultures every forty-eight hours in broth is generally sufficient to maintain the vegetative diplophase. Colonies produced by plating out are not sectored, the plates do not show colonial variants. However, when haplophase (single ascospore) cultures of any age are plated out, a variety of colonial variants appear on the plate or the giant colonies are sectored. These facts are consistent with the view that the large cells are diploid, thus minimizing the number of spontaneous mutations which may appear, while in the haplophase most mutants become apparent and are easily discovered.

d. When the diploid cells sporulate to produce haploid cells, there is genetic evidence of a reduction division (Winge and Laustsen, 78). Genetical analysis shows that a single pair of alleles responsible for the two different mating types is segregated at this meiosis. Two a and two α type haplophase cultures are usually obtained from the four single ascospore cultures (Lindegren and Lindegren, 46). There is also genetic evidence for the segregation of a gene-pair controlling fermentation of melibiose (Lindegren, Spiegelman, and Lindegren, 50) during the meiosis that precedes spore formation. Also, evidence proving that factors controlling cell shape may be segregated in a hybrid of *Saccharomyces bayanus* and *S. cerevisiae* (Lindegren, 34) has been accumulated in addition to that previously offered by Winge and Laustsen (81) in the balanced heterozygote, *Saccharomycodes ludwigii*.

e. Haploid cultures of a and α mating type have been paired, and the resulting diploid cultures in turn have been induced to sporulate, the haplophases have been tested and found again to fall into the a and α categories. Matings and tests for this character have been carried through four or five generations in several cases. Similarly, segregation of the gene-pair controlling melibiose fermentation has been observed, the segregants have been tested, mated, and segregation has again been observed in the succeeding generations. Pedigrees of three or four generations are available for many characters.

The above facts seem to prove conclusively that the terms haplophase and

diplophase can be used as definitely in speaking of yeasts as of organisms in which the cytological evidence is more complete The illegitimate diploids provide an exception which is quite familiar to the mycologist Copulations between haplophases which are usually incapable of copulating on genetical grounds were called "Durchbrechungskopulationen" by Brunswik (5) Copulations of this type frequently occur in single ascospore cultures and produce diploids which are homozygous for the *a* or *α* factors With rare exceptions these diploids sporulate poorly, and for this reason we have not studied them intensively A few single ascospore cultures sporulate well, and some produce large cells that are difficult to classify either as definite haplophases or illegitimate diploids However, the general rules laid down in the preceding discussion hold very well, and exceptions are not more frequent than in the higher plants where the phenomenon of alternation of generations (diploid and haploid) is well established

Haploids, Diploids, and Dicaryons

In the life cycles of other fungi, except yeasts, the only diploid nucleus is formed just preceding sporulation, and the life cycle is almost completely in the haplophase When the equivalent of a diplophase is required for genetical stability in such structures as toadstools, a dicaryon is produced This is a cell structure in which two genetically different haploid nuclei are paired in a single cell Since both nuclei function in the same cytoplasm, the resultant cell is practically, although not actually, diploid for the dominant genes suppress the recessives, resulting in the expression of only normal characters, and the suppression of all mutant characters for which the dicaryon is heterozygous (fig 1) Dicaryons or diploid nuclei are essential for organ formation because organ formation is based on the genetical stability of the building block, the cell Dicaryons, although they do not exist in yeasts, are the usual compromise of fungi in organ formation, they give genetical stability with considerable flexibility For example, when two dicaryons come into contact they may exchange nuclei to reconstitute new dicaryons without the necessity of first breaking down the nucleus by a meiotic mechanism The advantage which microörganisms derive from spending most of their existence in the haplophase consists in the fact that they are able to take advantage of every mutation which occurs, since the new gene begins to function immediately and by selection it may supplant the original forms Single mutant genes, however, cannot function in diploid cells (or in dicaryons) because the normal dominant gene suppresses the action of the mutant allele

Segregation, Mutation, and Recombination

In perfect yeasts the large, ellipsoidal vegetative cells, constituting the greater part of the life cycle, are truly diploid, thus setting these organisms apart from other fungi and ascomycetes in this very exceptional character Previously it was supposed that *S cerevisiae* produced its spores parthenogenetically Since haplophase vegetative cells are the rule among fungi, the large, ellipsoidal

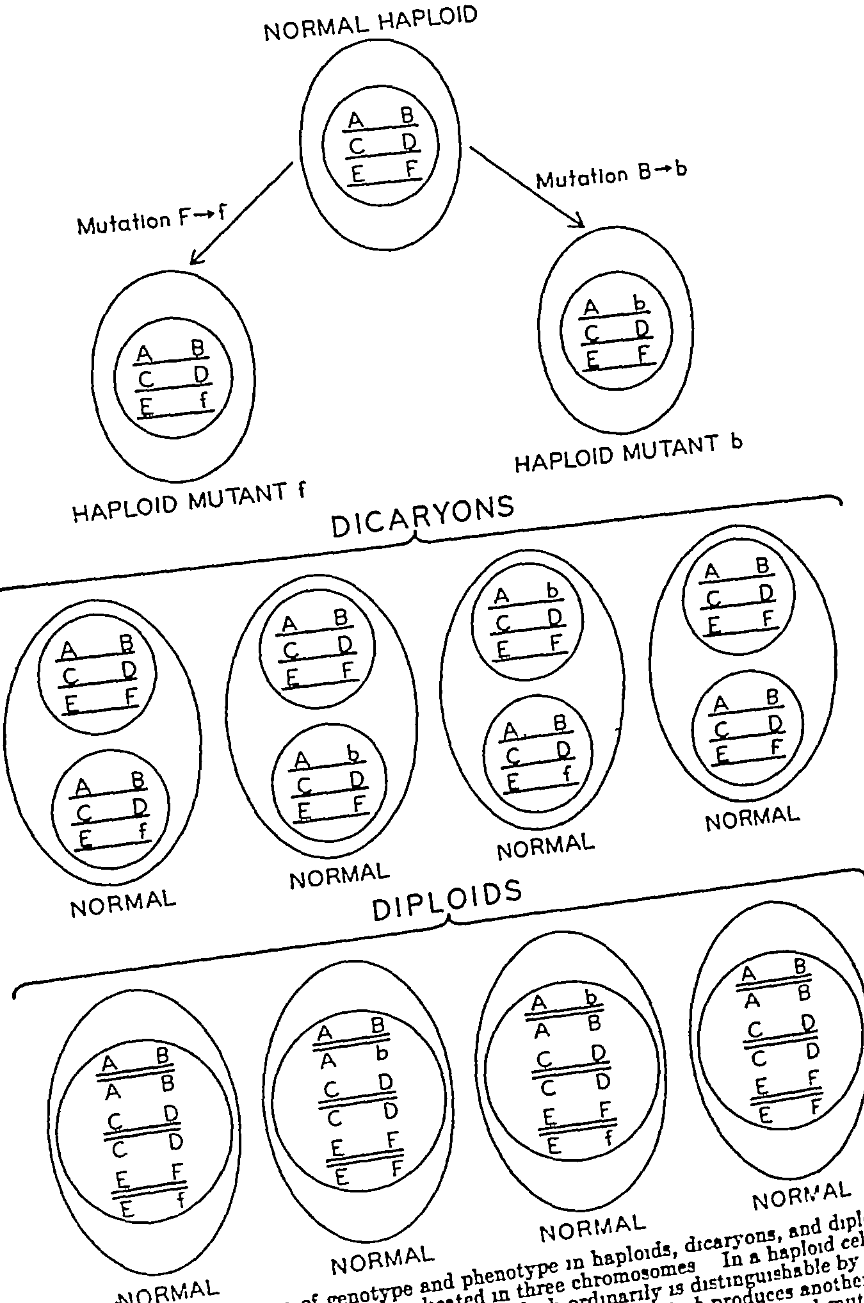

FIG 1 A comparison of genotype and phenotype in haploids, dicaryons, and diploids The genes *A-B*, *C-D*, and *E-F*, are indicated in three chromosomes In a haploid cell the mutation of *F* to *f* produces the new mutant *f* which ordinarily is distinguishable by some characteristic from its progenitor Similarly, a mutation of *B* to *b* produces another new mutant There are eight possible combinations of the normal, mutant *f*, and mutant *b* in dicaryons Six are represented and they are all normal since in every case the expression of the mutant genes is suppressed by a normal allele In diploid cells the same six combinations all appear normal for the same reason The greater flexibility of the dicaryon over the diploid lies in the fact that sexual fusions can occur without an intervening meiosis

vegetative cells were supposed to be haploid and spores were supposed to develop by an apogamous mechanism Kruis and Šatava (26) were the first to observe that the spores germinated to produce what they called "reduced" forms, which later fused to develop the standard, large ellipsoidal vegetative cells In their first joint paper, they did not give genetical significance to this phenomenon, apparently because Kruis was of the opinion that yeasts multiplied amitotically, but Šatava (65, 66) later suggested that the fused cells contained a single nucleus, and Winge and Laustsen (78, 81) showed that reduction of the nucleus to produce spores was accompanied by regular Mendelian segregation of morphological characters

Lindegren and Lindegren (46) demonstrated that the normal so-called legitimate diploid yeast cell is heterozygous for a single pair of mating type alleles, a/α, and that matings occur preferentially between two gametes of opposite mating type Two of the spores from each ascus belong to mating type a, while the other two belong to mating type α Each ascospore, when grown separately, produces a culture containing small, round haploid cells and hybridization occurs, producing large, dumb-bell shaped zygotes, when an a and an α culture are mixed in the same broth Copulations can be observed microscopically and large, diploid vegetative cells develop shortly thereafter

Haploid yeast cells are much smaller and more variable than diploid cells, varying more both from culture to culture and within a single culture than diploid cells These differences are also reflected in the colonies, the diploid colonies are larger and more uniform, while haploid cultures produce smaller colonies which are usually rough and generally show considerable variation (fig 2)

Segregation of genes occurs when the chromosomes are segregated at the reduction division of the diploid cells just prior to sporulation The haplophase originates by the reduction of the diplophase at spore formation, and the segregation of a heterozygote produces segregants of different genotypes Yeasts are extraordinarily heterozygous, and a great variation of colonial forms is obtained by the isolation of single ascospore cultures Each of the four spores formed in a single ascus is usually genetically different The haploid segregants are usually rough-colonied, smooth-colonied diploid cells usually produce only rough-colonied haploid segregants The segregant cultures also vary in their fermentative ability and in the size and shape of the haploid cells The type of cell aggregation is also characteristically different Haplophase clones generally tend to produce aggregated or agglutinated cells much more frequently than the diploid clones

Haplophase yeasts are nearly always inferior in their fermentative ability when compared quantitatively, or even qualitatively, to the diploid parent from which they originated, and many of them have lost certain specific characteristics For example, a single ascospore culture originating from *S cerevisiae* may be unable to ferment sucrose, or maltose, or galactose, although the original cultures fermented these sugars successfully

Mutation in the haplophase enormously increases the variation of colonial

forms, but the original segregant can generally be distinguished from the secondary mutants when the culture is plated out At first, the mutants are usually slow-growing and produce small, round colonies, but on transfer they become adapted and stabilized and their specific colonial character becomes apparent, distinguishing them from the original segregant *Schizosaccharomyces pombe*, which is normally unable to ferment galactose, can ferment this sugar if inoculations with cultures containing large numbers of spores are used. Mutations occurring in the haplophase are selected and propagated Most haplophase yeasts carried in the laboratory by serial transfer become sterile, i e , lose their ability to copulate with the opposite mating type In spite of the wide variety of types that mutation produces, the existing genotype of any cell limits its potentialities and the range of its possible variations This fact has been especially brought out in experiments aimed at adapting haplophases by selection *S cerevisiae* is unable to ferment melibiose, and prolonged exposure of haplophase cultures of *S cerevisiae* to melibiose failed to produce any mutants capable of fermenting this sugar A haplophase variant of *S cerevisiae*, incapable of fermenting galactose, could not be induced through a four-month period to produce mutants capable of fermenting galactose, although this strain produced an abundance of colonial variants during that same period Therefore, some "losses" as in the case of fertility, (see *Hybridization*) occur easily, while some "gains" as in the case of specific fermentative abilities, apparently do not occur at all Continued selection and plating of colonies often lead to the appearance of stable colonial variants which seem to have lost their capacity to produce other colonial types Some of these forms are round-celled and presumably haploid

Segregation and mutation produce a great variety of haploid gametes They all have the general characteristics of the species, but most copulations will produce recombinations different in a number of minor characters from the diploid from which they originated These facts are summarized in fig 2, showing that (1) segregation, (2) mutation, and (3) recombination, are genetical devices for producing variation in yeasts The large, ellipsoidal cells which produce a smooth colony on solid medium are usually heterozygous for a number of characters which are segregated at the reduction division and transmitted to four different ascospores which we usually designate arbitrarily as A, B, C, and D Each of these four spores generally produces a characteristically different colony the original segregant The haploid cells are usually round and much smaller than the original diploid cell When the haploid cells are plated out, a variety of colonial forms appear, indicating that many gene mutations have occurred When matings are made between the four clones in all combinations, the mating types are revealed. For example, in our specific case, pairing A × B and C × D fails to produce copulations, but the other four pairs all result in copulations, indicating that A and B are mating type *a* while C and D are mating type α The different designations for mating type are chosen arbitrarily Occasionally diploid cells appear in some of the single ascospore cultures, revealing that copulations had produced a diplophase homo-

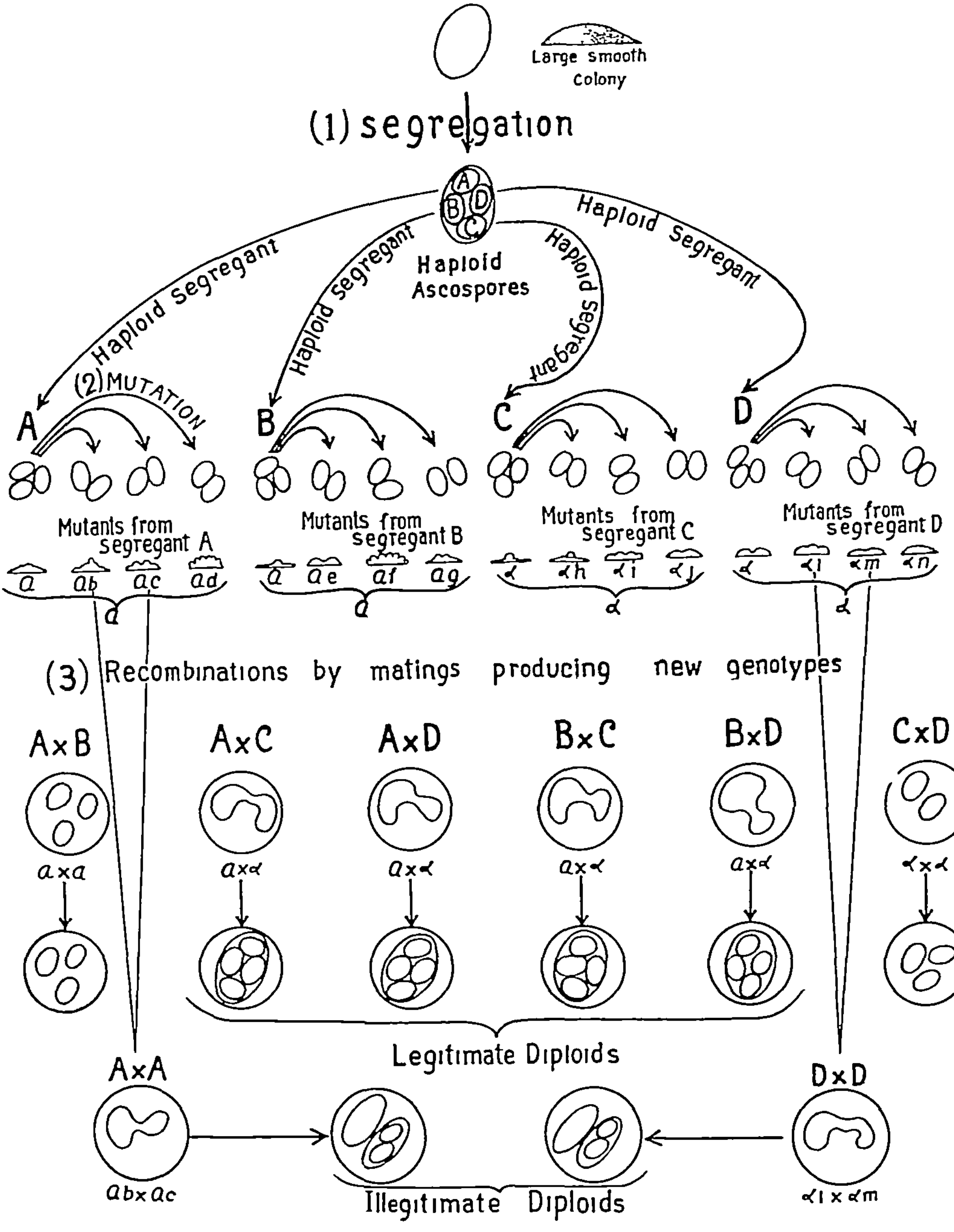

FIG. 2 A diagram showing the *genetical* mechanisms which produce variations in yeasts The legitimate diploid vegetative cell produces a large, smooth colony During spore formation, segregation occurs and four haploid spores are formed Since the original yeast is usually heterozygous for several loci each of the four segregants is usually genetically different and produces haploid cells which form a characteristic colony Plating these cells out on agar results in production from each segregant of a variety of rough colonies, distinguishable morphological mutants Three mutants are indicated in the figure but any number may be obtained depending on the persistence of the investigator Two of the segregants in this case from spores A and *B* are of sex *a*, while the other two from spores C and *D* are of sex *α* Whenever a suspension of cells from an *a* and an *α* clone are mixed, copulations occur as indicated by the diagram These produce diploid cells capable of producing viable four spored asci and are classified as legitimate diploids Most of these legitimate diploids may be genetically different from the original diploid genotype since many of the recombinations after segregation may be homozygous for many genes for which the original cell was heterozygous Illegitimate diploids may be produced by copulations between different cells or mutants of an original single ascospore culture These are characterized by smaller diploid cells and less viable, generally two spored asci

FIG 3 PHOTOGRAPH OF THE ASCOSPORES OF SACCHAROMYCES CEREVISIAE SHOWING TWO AND FOUR-SPORED ASCI

zygous for the mating type alleles The illegitimate diploid cells are usually somewhat smaller than the legitimate a/α diploids

Legitimate (a/α) diploids generally produce abundant and viable ascospores (fig 3), while the corresponding homozygous (illegitimate, a/a, α/α) diploids generally sporulate less vigorously and produce fewer and less viable ascospores The asci are often two-spored rather than three- or four-spored

The Rough Colony

The roughness of colonies grown on solid medium is a useful, diagnostic character in differentiating various cultures It apparently depends basically on the fact that the cells cohere to form a specific pattern, due to the manner in which they bud and branch This basic pattern of aggregation is obvious in the microscopic examination of cells from broth cultures The extreme rough-type colonies usually produce specific "rosette" aggregations Winge (76) described the "figure eight" arrangement common in haploid cells prior to copulation After a bud has reached full size, two new buds appear (one from the mother and one from the daughter cell) near the point of union of the daughter and mother cell, producing a "four-leaf clover" effect Most "rosettes" appear to be a variation of this "figure eight" formation Many cultures make what appear to be homogeneous suspensions because the "rosettes" are too small to affect the turbid appearance produced when the culture is suspended in fluid medium or grown in broth, but the extremely rough colonies cannot be easily brought into a homogeneous suspension and when extremely rough-type yeasts are grown in liquid medium, the supernatant liquid is often *completely clear* This is a character much desired for wine yeasts, especially for champagne yeasts There is a basic pattern of cohesion even in extremely smooth cultures, for nearly all colonies show some distinctive topographical structure if grown on solid medium long enough to form a giant colony Conversely, when moderately rough colonies are sown heavily enough on agar to prevent the formation of large colonies, only smooth ones appear On an unevenly spread plate one finds an outer fringe of extremely rough, large colonies and a central group of small, smooth ones All belong to the same genotype but the rough character cannot come into expression until the colony attains considerable size

We have observed several hundred different clones of rough-colonied yeasts, and although each one is distinctive and recognizable and can be duplicated and recognized when transplanted, we have not thus far discovered any exact duplicates The range of variation is extremely great In addition to the fundamental "rosette" or budding pattern, differences in shapes and sizes of the cells affect the colonial form In all colonies the variation in cell size increases with age, generally in the direction of producing larger cells As a rule, rough colonies contain more elongate cells than smooth colonies, and part of the basis for extreme roughness is possibly the maintenance of end-to-end connections after cell division, which has been described so frequently in the genus *Bacillus*

All four cultures from the single ascospores isolated from a four-spored ascus originating from stable, smooth-colonied, wild-type diploid cultures of *S cerevisiae* are usually rough-colonied This proves that the genes differentiating rough from smooth colonies are recessive and several loci are involved The wild-type "opposite number" alleles of the mutant genes prevent them from coming into expression in the heterozygous wild-type diplophase Although we have dissected many asci from the same diploid cultures, practically no duplicate cultures have been found among the colonies grown from the single ascospores, which indicates that the diploid cell is heterozygous for a considerable number of mutant genes, and that many loci affect the characters lumped into the so-called "rough" class

The Life Cycle of Saccharomycodes ludwigii

Copulations between yeast ascospores were observed as early as 1889 when Hansen described the genus *Saccharomycodes* Four spores are found in each

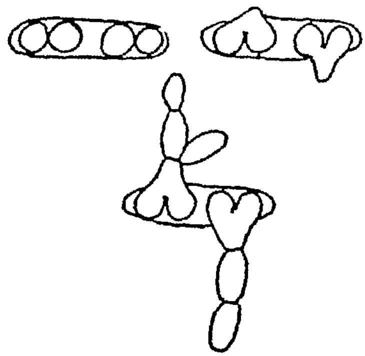

FIG 4 A DRAWING COPIED FROM WINGE AND LAUSTSEN SHOWING THE ARRANGEMENT OF SPORES IN THE ASCUS OF SACCHAROMYCODES LUDWIGII AND THE FORMATION OF SPORE ZYGOTES WITHIN THE ASCUS

ascus, two at each end of the cell The two paired spores always fuse (fig 4) within the ascus and germinate to produce a diploid cell which grows vegetatively until sporulation In this yeast the haplophase is transitory and exists only in the ascospores Winge and Laustsen (81) studied this organism in detail and found it to be a balanced heterozygote They showed that the haplophase could be cultivated by separating the spores from the ascus and that two spores in each ascus produced cells exhibiting normal growth, N, while two carried lethal genes, n, resulting in the early death of the haplophase cultures Segregation of another pair of alleles was observed simultaneously, which produced either long, L, or short, l, cell growth (fig 5) The two coherent spores are always complementary, if one is Nl, the other is nL, or if one is NL, the other is nl In each ascus the pair of coherent spores at one end is identical with the pair of coherent spores at the other end (fig 6) Winge confirmed these observations with several pedigrees and also determined the orientation of spindles in the ascus, finding that the spindle in Meiosis I is

longitudinal and centrally located, while the spindles of Meiosis II are located rather close together and overlap This is a variation of the mechanism in *Neurospora tetrasperma* which leads to the production of homothallic binucleate ascospores first described by Dodge (12) and analyzed further by Lindegren (36)

I have interpreted Winge and Laustsen's data according to the following scheme (fig 6) That the two genes are not linked is indicated by the fact that the four recombination types are found in equal proportions Therefore, both genes are on different chromosomes However, each is so close to the spindle attachment that segregation invariably occurs at the Meiosis I without crossing over After Meiosis I, the dyads in each nucleus will be either *NN, LL* and *nn, ll,* as in fig 6c, or *NN, ll* and *nn, LL,* as in fig 6C′ The fact that the spindles

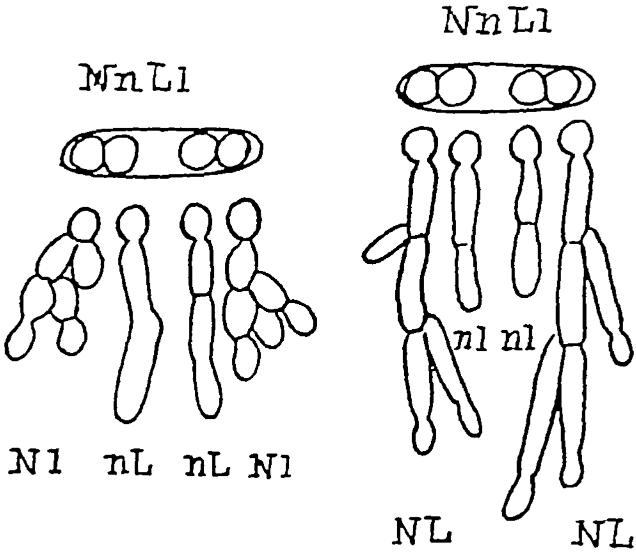

FIG 5 A DIAGRAM COPIED FROM WINGE AND LAUSTSEN SHOWING THE TWO TYPES OF SEGREGATION IN THE DOUBLY HETEROZYGOTIC SACCHAROMYCODES LUDWIGII

always overlap results in two possible final arrangements in the ascus These are the two arrangements that invariably occur

This analysis proves that the diploid *Saccharomycodes ludwigii* contains at least four chromosomes

II MATING TYPES

Although Winge (76) and Kruis and Šatava (26) both observed copulations between haplophase yeast cells derived from single ascospores, they obtained no evidence that the ability to copulate was under genetical control, in fact, Winge stated specifically that "fertilization does not consist in a union of cells that differ genotypically" In the cultures which he used the phenomenon probably was not so clear-cut as with our cultures, and even with our cultures many exceptions are encountered However, the facts which prove that a

single pair of alleles, arbitrarily designated a/α, control mating types are quite clear The following exceptions are found to the rule that matings usually occur between gametes of complementary mating types *a* There are occasional rare haplophase cultures of high fertility which copulate with other strains of the same mating type or with gametes derived from the same type (It is

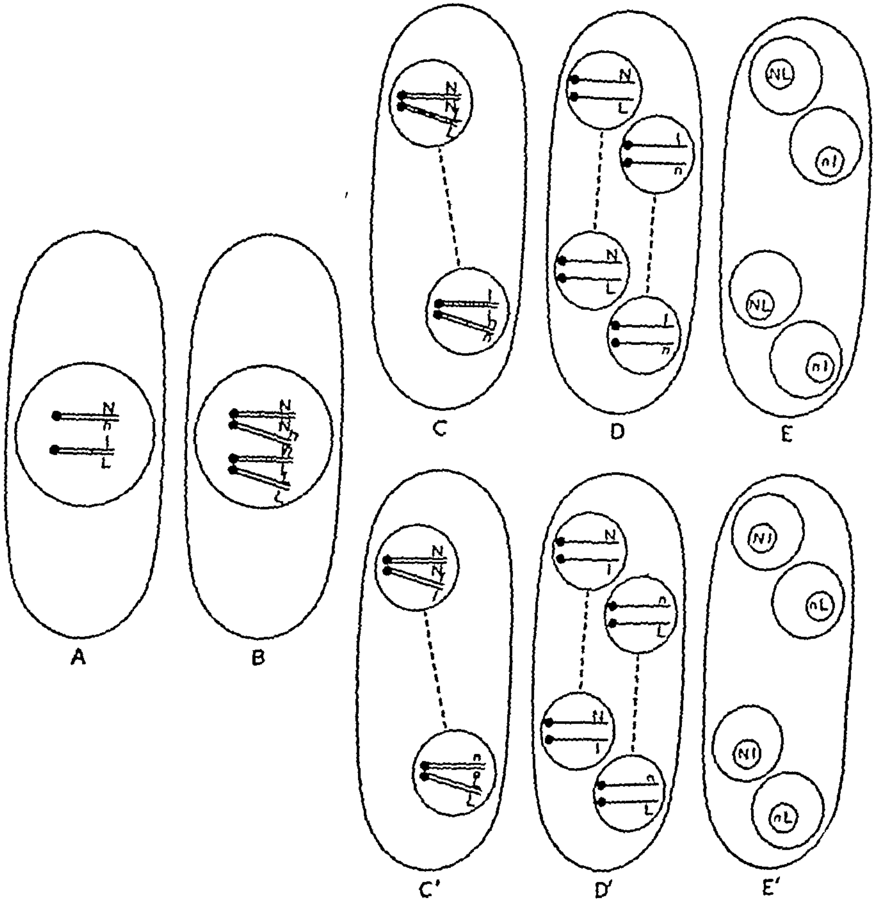

FIG 6 An interpretation of Winge and Laustsen's data on *Saccharomycodes ludwigii* showing why the doubly heterozygous diploid always produces two coherent spores which are likewise doubly heterozygous The genes are not linked, but are close to the centromere and segregation always occurs at meiosis I Depending on the orientation of the spindle, either C or C′ is produced The overlapping spindles in II place two complementary genotypes together in every case

from cultures of this type that the genus *Zygosaccharomyces* probably originated as a haplophase segregant of *S cerevisiae* Species of *Zygosaccharomyces* are usually rough-colonied, one of the common characteristics of haplophase segregants) *b* Some freshly isolated cultures are incapable of mating with any other gametes, and nearly all cultures, which are maintained by transfer, tend to

lose their fertility as they grow older (Round-celled *Torulopsideae* probably originated in this way) *c* Many single ascospore cultures produce diploid cells, but an extended analysis of pedigrees involving cultures derived from single ascospores revealed that the diplophases obtained in this manner were usually unable to produce viable ascospores, while diplophase cultures derived from four-spored asci usually produced viable four-spored asci

Conclusive proof of genetical control of mating type specificity was obtained by isolating the four single ascospore cultures from a single ascus and pairing them in all combinations (46) This experiment was repeated many times with substantially the same results indicated in fig 2

This experiment cannot be carried out with every set of single ascospore cultures obtained from a four-spored ascus because *a* single ascospore cultures are often sterile, *b* some cultures undergo illegitimate copulations very shortly after the ascospore germinates and diploid cells predominate in the derived cultures supplanting the haplophase, *c* more rarely the derived haplophase copulates with either *a* or α mating types, making it difficult to draw any conclusions from the data In almost any fresh haplophase culture from a single ascospore a few copulations may be observed, but when an active *a* and α mating is made, sometimes over half of the cells are copulating and it is very easy to distinguish this class from matings in which less than 1 per cent of the cells are fused Some diplophase cultures are extraordinarily fertile while others are quite infertile The L strain which we have studied extensively has very high fertility

The final proof of the genetical control of mating type specificity was obtained by mating 58 haplophase cultures from the L strain and a variety of other vigorous yeasts in all combinations (34) This experiment showed that *a* and α alleles were generally distributed throughout the species although they were often masked by sterility factors The α segregants of the L culture were a source of a large number of interstrain hybrids since they outcrossed rather easily with the *a* segregants of other strains The Lα segregants mated with the *a* segregants from other varieties almost as easily as they did with the L*a* segregants It was even possible to outcross the Lα segregants with *a* cultures from a number of other strains which were apparently sterile For example, B*a* and Bα cultures failed to copulate with each other while the Lα cultures easily mated with B*a* cultures

Mechanisms Insuring Cross-Fertilization

There are a variety of mechanisms in different plants and animals for insuring cross-fertilization, but all of these differ somewhat from that found in yeasts, the symbols a/α are given to this pair of alleles to indicate their uniqueness

a *Self-sterility alleles* Most hermaphroditic, flowering plants are self-sterile due to a genetic mechanism which prevents pollen shed by the flower from growing down the styles of the parent plant A mechanism that may be fundamentally similar prevents the sperms of an individual hermaphroditic sea squirt, *Ciona*, from fertilizing eggs produced by the ovaries of the same individual

b Sexual Dimorphism In higher animals and some plants sexual dimorphism insures cross-fertilization The genetic mechanism simply operates to reduce the probability of intersexes or hermaphrodites occurring

c Plus-Minus Factors This mechanism in *Rhizopus* is not a sexual mechanism because no unmistakeable sex organs are involved and therefore it cannot be called a self-sterility mechanism It is more precise to consider this a special case in which a single pair of alleles controls copulation

d Neurospora We formerly called the alleles in *Neurospora* plus-minus factors (38), but later work has shown that they resemble self-sterility alleles more closely than the factors found in *Rhizopus* Both plus and minus *thalli* contain both male and female sex organs and self-fertilization is prevented However, since the plus and minus *thalli* are both haploid and the zygote is invariably heterozygous for the same pair of plus-minus alleles, this mechanism differs considerably from the standard self-sterility mechanism found in flowering plants in which a series of multiple alleles exists and a great variety of heterozygotes abound

e Hymenomycetes The hymenomycete mechanism resembles the plus-minus *Rhizopus* mechanism rather closely since no obvious sex organs exist in these forms It differs in that two loci are often involved and that a multiple series of alleles at these loci may further complicate the picture

f Mating types The mechanism which assures cross-fertilization in the single-celled diploid *Paramecium* resembles the plus-minus mechanism found in fungi since no sex organs are present, but the heredity seems to be more complex The fact that the copulating cells are diploid is a still further difference from the most closely comparable fungal mechanism

Since no sex organs are present in *Saccharomyces*, Sonneborn's (67) and Jennings' (23) term "mating type" has been applied in the case of yeasts

I have used the letters a and α to distinguish the mating type alleles because the heterozygous a/α zygote is much more vigorous than either haploid parent, and it seems possible that these alleles may control the production of complementary, essential substances In this respect they may differ from conventional alleles which are usually indicated by large Roman letters Mating type alleles serve the function of facilitating cross-fertilization, for although illegitimate diploids occur by copulations between two a or two α haplophase gametes, the a/α gametes are generally much more vigorous than either the illegitimate or the haplophase cultures and thus outgrow the other forms After the reduction division has occurred, the four spores are almost invariably genetically different because of the extreme heterozygosis of the original diplophase The fact that usually two different ascospores or gametes derived from two different ascospores are mated practically insures the production of a recombination zygote different from the original diploid from which it was derived

The heterozygous zygotes have greater survival value than the homozygous zygotes, since the former produce viable ascospores while the ascospores produced by the latter are generally non-viable However, some exceptional

homozygous diploids produce viable four-spored asci. A culture of this type was described by Winge and Laustsen (82) in a study of a cytoplasmic effect of inbreeding in a homozygous yeast. It may have been their knowledge of the existence of a number of strains of this type that led them to the conclusion that mating type alleles did not exist in *Saccharomyces*.

Cross-fertilization has considerable survival value since it insures the incorporation of a variety of genes in each zygote and makes possible the production of a number of new recombinations. However, there are many organisms, such as the close-pollinated flowers, in which cross-fertilization does not occur, and inbreeding in this case does not appear to have yielded an inferior type, although it may have made the genus somewhat less variable and therefore less plastic or adaptable than cross-pollinated forms. I have described (37) copulation in a micrococcus in which cross-fertilization did not occur. In this case the zygote was invariably homozygous since it was formed by the fusion of two genetically identical gametes, which originated from the division of a single haploid nucleus. The fusion occurred within the cell after cell division, followed by the solution of the cross wall. However, this type of copulation may give rise to variation because it may provide an opportunity for chromosomal rearrangements to occur through some aberration of the mechanism. Autogamous copulation may represent an early evolutionary type of sexual mechanism resembling the parthenogenesis or apogamy that occasionally occurs in higher forms.

I prefer to define sex as Allen does, only in terms of true male and female sex organs. When we use this definition, mating type, self-sterility alleles, and plus-minus factors take on their true significance. They are not essential to the sexual mechanism but are simply means of assuring cross-fertilization. The fact that they may occur either in the absence of sex organs, as in the Hymenomycetes, or may be superimposed on true male and female sex organs as in Neurospora, prove that they are devices distinct from the sexual mechanism.

III. HYBRIDIZATION

Winge and Laustsen (79) produced many hybrid yeasts. They mated individual ascospores by placing them side by side with a manipulator and observed them until fusion occurred.

Lindegren and Lindegren (46) developed a new technique of hybridization. Matings were made simply by mixing mass transfers of haplophase cells in a fluid medium in a test tube. This method made it possible to study the genetical characteristics of the two haplophase parents (in ascospore matings both parents are lost by the fusion which forms the diploid). The micromanipulation was eliminated, except for that involving the original separation of the ascospores.

The haplophase culture developed from one spore can be used for an indefinitely large number of matings. This is particularly important since only occasional ascospores show a high degree of compatibility in matings. Haplophase cultures of desirable strains can be preserved by lyophilization. In this

process yeast cells are suspended in a protective colloid and the water removed by evaporation Tubes prepared in this manner are sealed and can then be stored in the dark at room temperature for many years without loss of viability of the cells When transferred to nutrient medium they are capable of initiating new cultures This is a helpful addition to the technique because lyophilized haplophase cultures retain their full copulative strength indefinitely Vegetative propagation of a haplophase culture is usually accompanied by mutations which reduce mating strength and transform the original type into a sexually impotent haploid

The advantage of alternation of generations in *S cerevisiae* was revealed by an extensive study of the hybrids obtained by making mixtures of mass transfers of haplophase cells Single diploid cells or single individual colonies selected after the zygotes had been produced revealed that a considerable variety of definitely different kinds of zygotes had been formed The hybrids were studied for growth rate or final density of growth in broth, but even with this simple classification five or six different kinds of zygotes were easily distinguishable following a single mating The haploid cells produce a variety of mutants, and apparently matings between these mutated gametes resulted in a corresponding variety of zygotes (fig 7) I have already pointed out that mutants produced by continued selection of cultures grown in the laboratory for a prolonged period (usually more than a year) are generally found to be sterile However, it appears that many of the mutations in the early stages do not prejudice the capacity of the gametes for copulation Prolonged competition and selection probably result in loss of fertility because the genes which insure fertility do not have a high survival value in competition with other rapidly growing mutants

These facts indicate that a moderately prolonged haplophase is a distinct advantage in *Saccharomyces* because it permits competition among the new mutants that arise in the haplophase, leading to selection of the most vigorous before copulations occur This mechanism may account for the fact that *Saccharomyces* is the most cosmopolitan species of yeast If the mating type strength is great, as in *Saccharomycodes,* fusions occur in the ascus without selection or competition between the gametes and the plasticity of the species is limited, for in the diplophase (which constitutes almost the entire life cycle in *Saccharomycodes*) mutations are not so readily selected. It is the relatively weak mating strength evidenced in *Saccharomyces* which makes an extension of the haplophase possible The greater vigor of the diplophase enables it to outgrow the residual haplophases The variety of genes affecting mating strength results in the production of a large variety of sterile haploids (which are essentially "blind alleys" in terms of the continuity of the species), and many of these haploids become stabilized in the form of the genus named *Torulopsis*

Selection and Hybridization Mutually Exclusive

Previous to the development of a system of hybridizing yeasts all improvements resulted from selection The selected cultures were often diploid and

because of this were relatively stable These variant cultures were probably produced by recombinations following spore formation and were either legitimate or illegitimate diploids Variants of this type might be picked up in a single selection However one can carry on a continuous selection with the unstable haplophase cultures and get improvement at each step, if a sufficiently large population is tested, and if the desired character is not stabilized by the

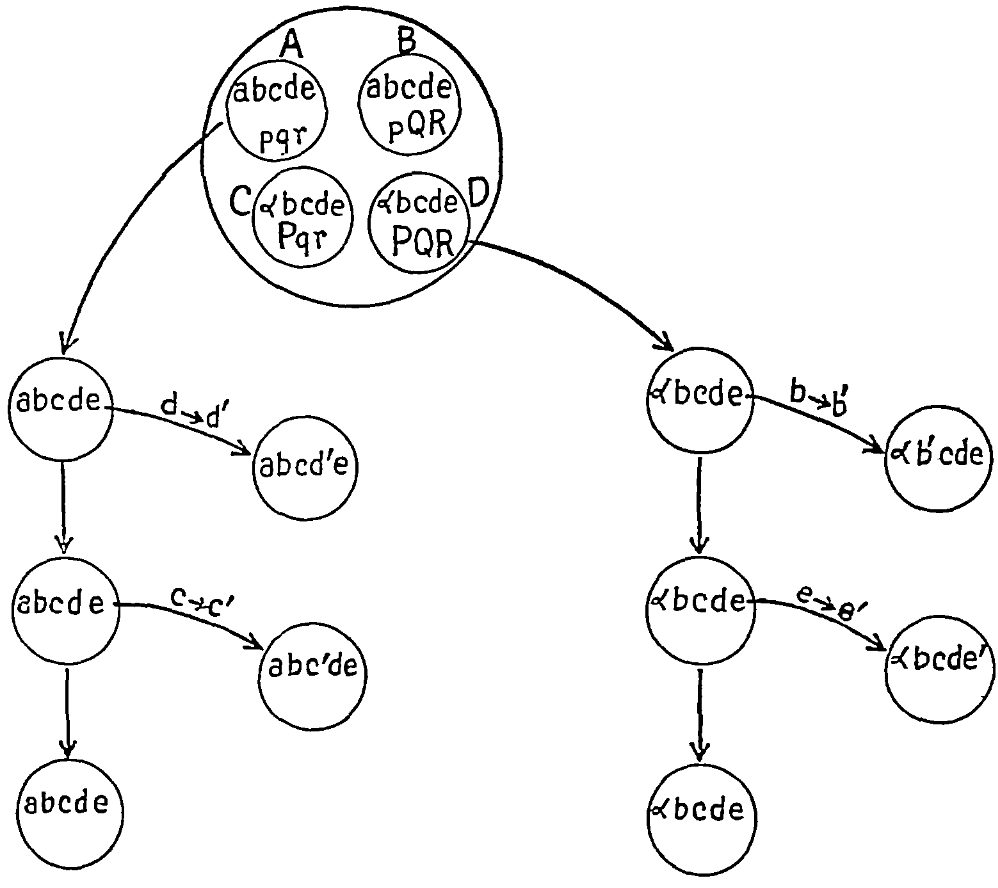

FIG 7 A diagram indicating how segregation followed by mutation in the haplophase can produce a variety of gametes If the original diploid cell is homozygous for *bcde* and heterozygous for *apqr*/*αPQR*, the four ascospores may have the compositions indicated In a clone isolated from a single *abcdepqr* ascospore (mating type *a*), a mutation of *d* to *d′* produces a mutant *d′* (*abcd′epqr*) and a mutation of *c* to *c′* produces mutant *c′* (*abc′depqr*) In a clone isolated from a single *αbcdePQR* ascospore (mating type *α*), a mutation of *b* to *b′* produces an *αb′cdePQR* clone while a mutation of *e* to *e′* produces an *αbcde′PQR* clone When cells from the two original culture tubes are mixed, nine new recombinations may be produced

gene complex present in the cell Such continued selection generally involves so much mutation that the resulting culture is sterile and incapable of hybridizing Intensive selection and hybridization are, therefore, mutually exclusive procedures Matings can only be made between haplophase cultures that have been recently derived from single ascospore cultures Since haploid yeast cultures become sterile when subcultured vigorously, the test

for mating type can be performed only with single-ascospore cultures obtained from freshly isolated spores The genetical differentiation between the *a* and *α* types limits the capacity of haplophase cells for copulation, except with members of the complementary mating type

Generation Time

A short generation time is an advantage in genetical studies The following schedule reveals the minimum time intervals involved in yeast breeding

Day	*Program*
0	Isolation of ascopores
1	Transfer of haplophase colony to agar
2	Mating of haplophase with tested strains
3	Observation of positive matings and transfer to pre sporulation medium
5	Transfer to gypsum
6	Isolation of ascopores

IV CULTURAL VARIABILITY AND STABILITY

Many of the physiological and morphological characteristics of yeasts and bacteria are remarkably stable in the hands of experienced investigators Comparable quantitative results on fermentation, infectivity, rates of growth, and a variety of other measurable characteristics are obtained regularly Many of the workers who have had experiences of this type are inclined to minimize the importance or the frequency of variation among microörganisms On the other hand, investigators who are interested primarily in variation encounter a surprising lack of uniformity in all the quantitative or qualitative characters which come under observation Differences in the results obtained by investigators with different interests probably result directly from the use of different techniques in maintaining and transferring cultures Any given clone, whether haploid or diploid, is generally quite uniform in appearance and character, provided mass transplants are consistently used For example, the standard practice in handling yeasts in industry is to grow the cultures in broth and make transfers by pipetting most of the cells from an old broth tube to a fresh broth tube Under these conditions, the predominant genotype is maintained by the transfer and the culture usually duplicates very closely the performance of the parent strain Even if a rather high frequency of variation occurs, the effects of the variant forms will be swamped out by the many billions of the predominant type of cell and will affect the quantitative results only slightly In the same way, bacteriologists accustomed to obtain duplicating results in the study of specific quantitative characteristics, use mass transplants of bacteria Usually a full loop of cells is removed from a slant and streaked on a second slant or inoculated into broth By contrast, students of variation streak culture plates very lightly, aiming at a large number of single colonies, or inoculate broth with single cells Investigators using the latter techniques almost invariably uncover a great deal of variation in any microörganism The amount of varia-

tion is multiplied in the case of yeasts when single ascospores are isolated following genetical segregation. One who is convinced that yeasts are quite stable will be amazed at the degree of variation that results when he simply takes an old culture which has sporulated well, suspends the cells in water, heats the suspension to about 60 C for five or ten minutes and then plates very lightly on agar. I believe that these two differences in technical approach are the main basis for a great deal of disagreement with regard to the amount of variation that is encountered in routine laboratory manipulations by competent workers in microbiology.

The Effect of Volume Relations on the Variability of the Haplophase

Haplophase cells multiply in the medium to produce large numbers of genetically and morphologically different forms. Selection pressure is more effective in the haplophase of single-celled microörganisms than in any other biological form. Free living cells exist in a constantly changing medium, and each new variant is tested for its survival value in competition with its predecessors. Since the population existing at any given time has been selected only with regard to its ability to compete under previously existing conditions, each new variant has a chance to outgrow the earlier forms. In a liquid medium containing a small inoculum, there is little competition at the first stage of growth because the "biological space" available to each cell is large and its by-products which function to limit growth, are quickly diluted. The available nutrients are also at a maximum. Under these conditions, a variety of forms accumulates, because there is opportunity for even the weakest to multiply. Following the early "era of good feeling" there is a phase of intense competition in which a few predominant types emerge to grow rapidly and finally come to a maximum. This prevailing type is particularly adapted to the substrate conditions during the logarithmic phase, and it succeeds in overwhelming other types by sheer weight of numbers during the latter stages of the logarithmic phase. However, in the very late stages of growth, the predominant form either is prevented from growing further or begins to die off. New conditions obtain which are especially adverse to the organisms predominating in the logarithmic phase because the peculiar complex of by-products characteristic of the predominant form is at a maximum.

The mutation rate need not be unusually high to produce many variants under these conditions. Each haplophase culture is a complex mixture of different morphological and biochemical types, and the wide range of variation makes it difficult to characterize haplophase cultures precisely. Haplophase cells generally sporulate sparsely and are less vigorous and less efficient in the utilization of carbohydrates than the corresponding diplophase. Morphological and biochemical studies lead to variable results when performed with haplophase cultures. It is possible, nevertheless, to obtain an idea of "average" biochemical potentialities of a haplophase if the culture is maintained by massive transfers which are made frequently.

There are two phases in the adaptation of a haplophase culture to an originally unfermentable carbohydrate, *a*, the *selection of a specific new mutant* by a shift in the populations and *b*, the adaptation of this mutant by exposure to the carbohydrate The first step concerns the genes, the second concerns the cytoplasm The haplophase complex seems to have a more or less equilibrium composition, and tests, for example, of the time of adaptation to galactose of a haploid culture yield data showing that an exposure of a few hours to several days transforms different subcultures of one originally pure single ascospore culture to a fermenting type of organism

In the diplophase (where mutations cannot express themselves), there is no shift in population, provided sporulation is prevented, and adaptation occurs by the interaction of the carbohydrate with the cytoplasm Diplophase cultures, under these circumstances, are either adaptable or non-adaptable, and the adaptation time can be determined to within a few minutes

The variability of the haplophase makes it the organism of choice for the selection of variants Haplophase yeasts are capable of a wide range of adaptation and specialization with regard to biochemical reactions, especially when freshly isolated from the ascospore However, after several months' selection, some cultures have been obtained with extremely specific, apparently stabilized colonial characteristics

With organisms as variable as the haplophase yeast, it is clear that single cell isolates will produce an enormous degree of variability and that commercial production with yeasts of this type would be hazardous If, however, an exceptionally desirable haplophase yeast is encountered, it is possible to use it in industry by growing the cells in a series of receptacles of gradually increasing size by consideration of the kinetics of competition In spite of this high degree of variability, it is possible to utilize these haplophase cultures in industry by consideration of the relation of the volume of the available medium to the growth rate It is important to realize that the mutation rate is beyond the control of the operator, but even if it is not possible to reduce the mutation rate, it is possible to minimize the initial "era of good feeling" by reducing the volume of available nutrients so that the forces of competition are brought into play quickly This will minimize the period of relatively unrestrained multiplication, in which large numbers of a weaker type of cell manage to gain a foothold When a chosen mutant has been found to have some especially desirable character, a number of cells can first be grown in a single drop This entire drop can then be transferred while growth is in the logarithmic phase to a few milliliters of medium, bringing the forces of competition into play early in the growth cycle The next transfer should be made by transferring the entire contents of the previous culture into a quantity of medium likewise designed to eliminate the early non-competitive phase, and each succeeding transfer should be handled in the same way Since the increase in growth is logarithmic, a large number of serial transfers will not be required, but they must bear such a relation to the growth rate that the logarithmic phase is constantly maintained This process can be worked out empirically once the mechanism of crowding is understood

V SPORES AND SPORULATION

The production of spores by *S cerevisiae* is controlled both by the genetical composition of the culture and by the nutrient on which the culture has been grown previous to transfer to gypsum Yeasts of the proper genetical composition grown on a special pre-sporulation medium and transferred to gypsum slants usually sporulate within twenty-four hours The percentage of cells sporulating following growth on this medium is rather high, occasionally 90 per cent of the cells form spores Relatively slight changes in the medium cause considerable differences in the percentages of spores produced The effect on spore production of growth on a medium containing a large amount of natural nutrients as contrasted to the effects on spore production of growth on a synthetic medium or a poor nutrient medium, suggests that sporulation is affected by a variety of accessory substances, which are either necessary in large concentrations or may not yet have been identified Occasionally, diploid cells sporulate directly on a pre-sporulation medium, but usually it is necessary to transfer them to gypsum slants to complete the process

Sporulation in yeasts was probably first studied by de Seynes (10) who reported that round spores found "in the surface of the water" germinated to produce elongate cells in a mixture of wine and water, but that these elongate cells produced spores again when transferred to a more dilute medium Rees (60) found that when yeast was planted on the cut surface of various vegetables (cooked or raw) growth continued until the fourth day, when budding stopped On the fifth day, the vacuoles in the cell disappeared and the protoplasm became coarsely granular Spore formation occurred regularly on the sixth day Spores also appeared on the sixth day whenever fresh yeast from beer vats or wine must was transferred to the cut surface of carrot or potato Rees did not find spores in old lagering vats or in compressed yeasts, but washed yeasts from these sources placed in a beaker in a layer approximately 4 mm thick and protected from dust sporulated abundantly in about three weeks

Welten (75) challenged the view that starvation was essential to sporulation He found that on prune extract agar, yeasts sporulated well He even doubted the necessity for oxygen, since in his experiments, colonies imbedded in the agar sporulated as well as those on the surface. He found that yeasts grown in pear extract or beer wort did not sporulate so well as those grown on prune extract agar But sporulation occurred when the washed yeast grown on prune extract was placed on glass plates, filter paper, or sterile washed agar slopes, if a drop of prune extract were added If no prune extract was added, no spores were produced Welten's work proved that starvation alone is not the complete explanation of the phenomenon He showed that acidity of the medium in which sporulation occurred was important, no spores occurring in an alkaline milieu Welten also found that more spores were produced in concentrated than in dilute prune extract, also that a small amount of $MgSO_4$ aided sporulation Young cells were not essential, those three to four days old sporulated better than those one to two days old

Mrak, Phaff and Douglas (58) discovered that many yeasts sporulated well

on slopes of an agar medium containing a mixture of vegetable (cucumber, beet, potato, and carrot) extracts

A New Medium for Inducing Sporulation

Genetical analysis requires an abundance of large, viable four-spored asci Lindegren and Lindegren (47) developed the following pre-sporulation medium

Beet (leaves) extract	10 ml
Beet (root) extract	20 ml
Apricot juice	35 ml
Grape juice	16 5 ml
Yeast (dried)	2 g
Glycerin	2 5 ml
Agar	3 g
$CaCO_3$	1 g
Water to a final volume of	100 ml

The mixture was steamed for ten minutes and tubed. The tubes were sterilized at 15 pounds pressure for twenty minutes and slanted. Most strains of yeast will produce spores directly on the slants if allowed to grow for a few weeks However, if spores are needed sooner, transfer to gypsum is necessary

Engel's (13) gypsum block method was replaced by the method of Graham and Hastings (15) A mixture of plaster of Paris and water (100 g of each), is poured into test tubes and solidified in a slanting position These slants are dried at 50 C for twenty-four hours and autoclaved

About 1 ml of sterilized water is poured over a three-day growth of yeast on the pre-sporulation medium and allowed to stand 10 minutes, then a thick suspension is made by stirring the yeast cells around in the supernatant fluid The yeast suspension is taken up in a pipette and poured over the upper part of the gypsum slant About 3 ml of sterile water containing enough acetic acid to bring the pH to 4 is pipetted into the lower half of the gypsum slant The inoculated gypsum slants are incubated one to two days at 25 C

Variations in the Number of Ascopores in an Ascus

Ideally, each ascus contains four ascospores, but this ideal is not invariably attained, in fact, one much more frequently encounters two- and three-spored asci than four-spored asci, while one-spored asci abound in some cultures and on rare occasions one finds asci with more than four spores Since the reduction division always produces four basic nuclei, less than four spores in the ascus of an ascomycete is generally interpreted as the result of disintegration of nuclei A two-spored ascus would presumably result from the disintegration of two nuclei and the incorporation of the remaining two into ascospores Ascospores may disintegrate as a result of competition within the ascus An example of this type was analyzed genetically by Lindegren (35) in *Neurospora* Yeasts that are capable of producing four-spored asci when properly nourished will produce almost exclusively two-spored asci on potato agar, suggesting that the

two eliminated haploid cells are capable of developing only when they are fed on a rich, natural medium.

Some illegitimate cultures produce large numbers of viable one-spored asci, while in these cultures the spores from asci with more than one spore are non-viable. Asci from a culture of this type were analyzed by Lindegren and Lindegren (47). The one-spored asci were of special interest because they were found to germinate directly to produce diploid cells. The diploid cells were indistinguishable from the original culture, proving that spore formation occurred without reduction. Therefore, large, one-spored asci contain spores with a diploid nucleus that has not undergone reduction (possibly by monaster formation), a diploid nucleus is simply enclosed in a spore wall. The general low viability of the haploid spores from two-spored asci originating from homozygous diploid cells may result from the aberrations of the reduction division in homozygous diploids, suggesting the possibility that homozygosis is usually prejudicial to a regular meiosis.

Cytoplasmic Degeneration in Homozygous Strains

Winge and Laustsen (82) studied a strain of homozygous *S. cerevisiae* which produced viable four-spored asci. Diploid cells were produced either by fusion of two of the gametes after multiplication of the haplophases, or by a fusion of the first cell budded from the ascospore with the original ascospore. Diploid forms produced by the fusion of two cells were able to perpetuate the homozygous race and produced asci containing four haploid, viable ascospores. An alternative mechanism for producing the diploid cells involved the direct germination of a diploid (zygote) cell from a haploid ascospore (an ascospore from a four-spored ascus). The latter case was interpreted as follows: a nuclear division occurred within the ascospore in the absence of a division of the cytoplasm, this resulted in the production of a diploid nucleus carried in a cytoplasm whose constituents (which apparently divide normally at each cell division) had not been able to reproduce themselves (fig. 8). The resultant deficiency was indicated by the fact that the ascospores of these diploid cells were of low viability. In contrast the ascospores of the homozygous strain from which they had been derived were highly viable. This constitutes proof of a mechanism of cytoplasmic inheritance in which a set of plastogenes divide regularly at each nuclear division. If this division were irregular, one would anticipate the damage which the cell had suffered could be repaired in time.

We have evidence supporting Winge and Laustsen's work. We have observed that haploid ascospores (spores isolated from four-spored asci) which germinate directly as diploid cells, usually produce only a small colony which soon ceases to grow and apparently dies since transfers from the colony do not develop.

VI. SPECIATION IN YEASTS

It has often been pointed out that there is no satisfactory definition of a species. This applies even more specifically in yeasts and fungi than in higher

forms Winge and Laustsen, and Šatava, have suggested that *Torulopsis* and *Zygosaccharomyces* must have been derived from some species of *Saccharomyces* and can probably be looked upon as derivatives of the parent species It is not known at present which species of *Saccharomyces* gave rise to them A great variety of other genera probably also take their origin from *Saccharomyces*

The original genus produces four viable ascospores and is therefore a *perfect* form The haploid ascospores produce gametes with relatively weak copulative strength and the mating type alleles are modified by a number of sterility genes This results in an extraordinarily plastic species Segregation of the genes at spore formation produces a variety of haploid genotypes which compete among themselves by vegetative reproduction previous to copulation Mutations can occur in the gametes produced from the ascospores during the period of vegetative reproduction before copulation, and selection acts to elimi-

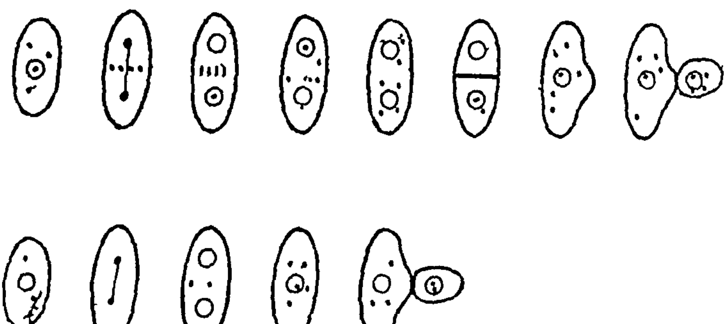

FIG 8 A diagram copied from Winge and Laustsen (82) giving a hypothetical explanation suggesting how a diploid cell produced by the fusion of two homozygous gametes, after the cell division has permitted chondriosomes to divide (upper row), may differ from a diploid cell produced by the fusion of two nuclei which have been produced by a mechanism in which nuclear division has occurred but chondriosomal division has been suppressed

nate the less vigorous or the poorly adapted gametes This increases the variety of forms which become adapted to specific environmental niches The weak copulative strength delays copulation until the variety of gametes has been multiplied by segregation and mutation The mating type alleles insure cross-fertilization and the production of heterozygous diploids Copulations occur between the predominant forms in greatest abundance enabling the best adapted to take over the living space in which they originated At the same time, copulations between less well adapted forms, which may be able to achieve eminence subsequently in another environment, are not completely excluded

Besides legitimate diploids other clearly differentiated forms may also be produced by the mechanism *a* If the mating type alleles become linked to a gene-pair containing one lethal and one normal allele, selection will increase the gene-pair containing one lethal and one normal allele, heterozygous for both copulative strength Finally, a balanced heterozygote, heterozygous for both the mating type alleles and the lethal-normal gene-pair, will develop in a manner similar to that found in *Saccharomycodes ludwigii* *b* Stabilized haplophases

also will arise from *Saccharomyces* by segregation of a haplophase incapable of copulation, but otherwise well adapted, resulting in forms like *Torulopsis c*. In addition, segregation of genes for high fertility, readily capable of producing illegitimate zygotes, and able to sporulate easily, will lead to the development of *Zygosaccharomyces* as an offshoot from the main genus *Saccharomyces*. In fact Winge and Laustsen were able to produce an intergeneric hybrid between *Saccharomyces* and *Zygosaccharomyces*, indicating that these genera are closely related. The evidence at present available disproves Guilliermond's contention that these genera are distantly related. It is notable that the *Zygosaccharomyces* abound in media of high sugar concentrations, to which they seem extraordinarily well adapted, while *Torulopsis* is adapted to dilute, well-aerated media. Both of these genera are weaker fermentative variants of the original actively fermentative *Saccharomyces*.

Schizosaccharomyces is easily differentiated from *Saccharomyces* and falls into an entirely different category. Its spores stain blue with iodine, while those of *Saccharomyces* stain yellow. Its cells divide by a regular binary transverse fission which is distinct from the unique mechanism of budding characteristic of *Saccharomyces*. The cultures of *Schizosaccharomyces* available in our laboratory are homothallic and homozygous, i.e., haploid cells are first produced in profusion, those arising from a single ascospore fuse to produce a diploid from which four- or eight-spored asci are readily obtained. There is no evidence for the existence of mating type alleles. All single ascospore cultures produce an abundance of asci containing viable ascospores. This seems to be somewhat at variance with the yeasts originally described by Beijerinck (3, 4). He obtained many non-sporulating cultures, a fact suggesting that some stabilized haplophases may also stem from *Schizosaccharomyces*. In maintaining *Schizosaccharomyces* in the laboratory, the selection of sporulating forms would naturally occur, because this is the criterion of the species and concentration on this character may have resulted in the isolation and perfection of a homothallic strain.

The Phylogenetical Significance of Biochemical Criteria

Morphological characters are probably preferable to biochemical or fermentative characters in tracing phylogenies because many of the haplophase segregrants from powerfully fermenting strains are singularly weak in fermentative ability as was shown by Lindegren, Spiegelman and Lindegren (51). The diplophase *S. cerevisiae* can ferment glucose, levulose, mannose, galactose, sucrose, and maltose, while many haplophases originating from this species have been obtained which had lost the ability to ferment one or more of these sugars, although they were generally able to oxidize them. Some of these segregants were unable to ferment even glucose although they could utilize it by oxidation. The loss of ability to ferment is a very common character resulting from either segregation or mutation.

Saccharomyces fragilis is differentiated from *S. cerevisiae* by the fact that the former ferments lactose but not maltose, while the reverse is true with *S. cere-*

visiae The ability to ferment either lactose or maltose is generally a mutually exclusive character, only *Brettanomyces* can ferment both These data suggest that the same gene controls both fermentations If this be true, the lactose fermenters may arise from maltose fermenters by a single mutation in the haplophase The species of *S fragilis* which we have studied have kidney-shaped weakly viable spores *S fragilis* may have originated from single ascospore cultures from some perfect *Saccharomyces* developing in milk. Mutation at the maltose locus to a gene capable of initiating the fermentation of lactose could make it possible for the culture to multiply and predominate in milk An illegitimate copulation between the haplophase in the milk might have resulted in the diplophase of *S fragilis* *Zygosaccharomyces lactis* probably arose in a similar manner, except that in this case a highly copulative haplophase occupies the predominant portion of the life cycle

VII CYTOLOGY

The yeast cell contains more identifiable organelles than many other plant cells, but there has been little agreement on the question of which of these organelles correspond to the conventional chromosomes and nuclei of higher plants The observations of Henneberg (22) and Wager and Peniston (74) are, in my opinion, the most complete, and my own observations follow these authorities closely Wager and Peniston's interpretation was limited by contemporary concepts of cell structure, but their drawings reveal an organization easily understandable in terms of modern concepts of the nucleus They show that the yeast nucleus has a structure similar to that described by Harper (21) for the ascomycete, Phyllactinia Attached to one side of the nuclear vacuole is a smaller body which is not ordinarily visible in the living cell This structure is visible in iodine-potassium iodide preparations (fig 9) and corresponds to the centriole in Phyllactinia The nuclei of many fungi, especially the hymenomycetes, have a notably eccentric appearance due to the fact that the centriole, a large body with a strong affinity for hematoxylin, is attached to the apparently empty nuclear vacuole Wager and Peniston described the chromosomes polarized to the centriole, exactly as they are in the higher ascomycetes Guilliermond (16, 17, 18) probably mistook the centriole for the nucleus because it divides at each mitosis, shows internal structure, and retains hematoxylin rather firmly However, this body is much too dense to be a nucleus The nucleus is invariably a structure containing a dilute nuclear sap surrounded by a membrane The chromosomes are suspended in the dilute nuclear sap which stains poorly or not at all, in contrast to the cytoplasm which often stains very heavily The body attached to the nuclear vacuole in yeasts is an extremely dense, heavily staining body, thus excluding the possibility that it is the nucleus

Volutin, or metachromatin, was originally defined by Meyer (57) as a substance which stained dense blue with methylene blue, but did not destain with 1 per cent sulfuric acid There does not seem to be a general agreement on the identity of volutin in yeast cells Wager and Peniston called particles in the

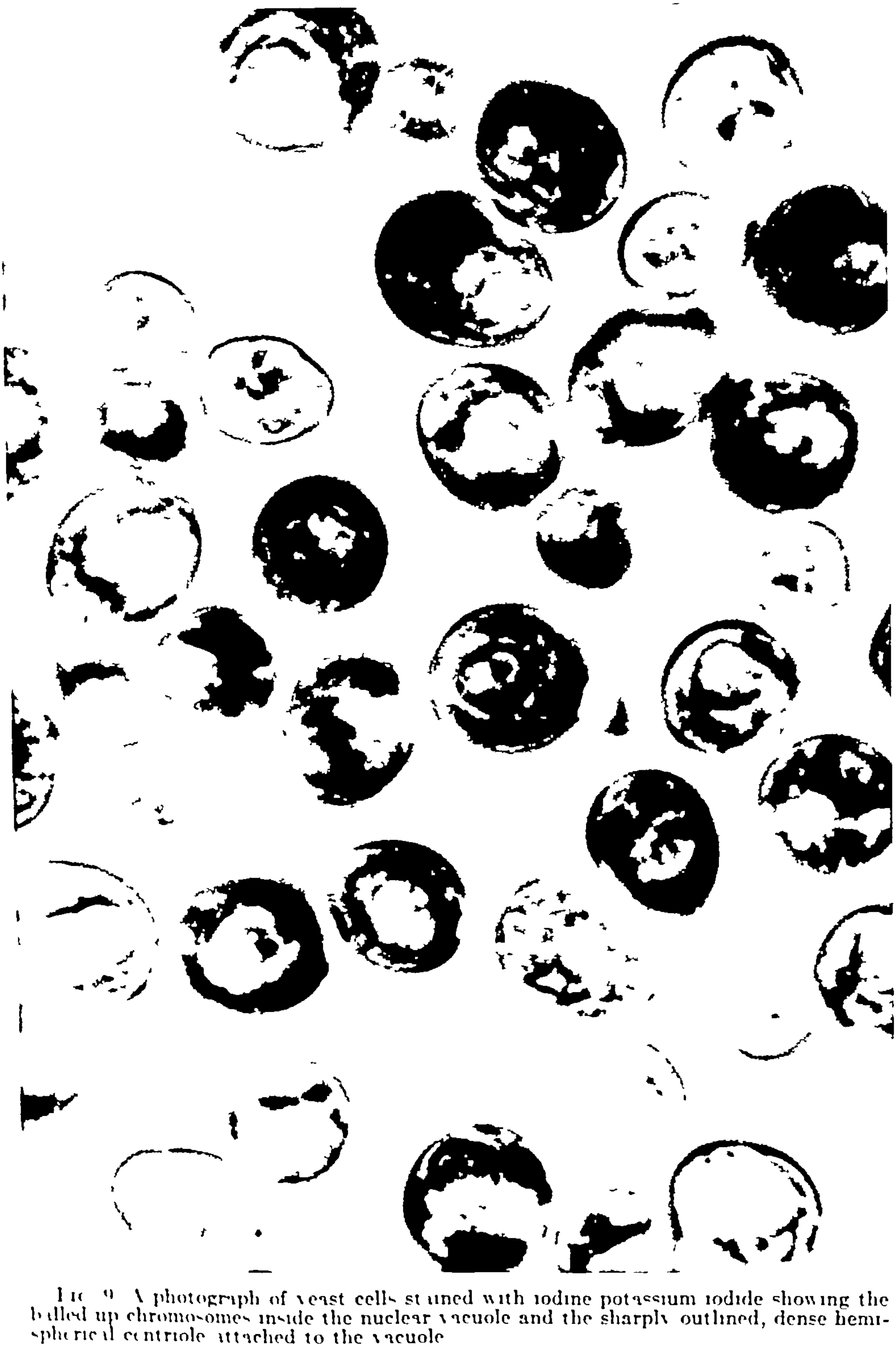

FIG. 9. A photograph of yeast cells stained with iodine potassium iodide showing the balled up chromosomes inside the nuclear vacuole and the sharply outlined, dense hemispherical centriole attached to the vacuole.

cytoplasm which stained red with methylene blue, volutin, and disagreed sharply with Guilliermond on the distribution of volutin and chromatin in the yeast cell Henneberg seems to have followed Meyer's technique closely and states that volutin is found inside the vacuole He points out that volutin bodies inside the vacuole appear in a variety of forms Sometimes they appear as long, slender threads, sometimes they are short, stocky, almost cylindrical bodies, sometimes they appear as six to twelve slender threads radiating from the centriole, and sometimes they exist as a rather large number (forty to fifty) of discrete dots He did not consider them to be chromosomes, as a matter of fact, he agreed with Guilliermond in considering the centriole to be the nucleus Wager and Peniston also found what they called "chromatin" in the vacuole in the form of about fifty small, discrete bodies

Badian (2) developed an exceedingly effective stain for bacteria and fungi He killed the cells with osmic vapors, stained with methylene blue, and destained with eosin He studied mitosis and meiosis in *S cerevisiae,* and *stated that the cells contained two chromosomes which divided by longitudinal* splitting However, his figures show that the so-called chromosomes always pull apart finally by thinning out at the middle and the final separation is by a crude, transverse fission Furthermore, he stated that the haploid chromosomes fuse end to end to form the diplophase, rather than associating to form a pair of chromosomes according to the usual method If his conception is correct, the number of chromosomes in haplophase and in diplophase would be the same

Volutin Chromosomes

The structure which I have called the centriole contains two rod-shaped bodies which stain well with aceto-orcein and divide by a crude transverse fission (fig 10) They are the only bodies in the cell which take aceto-orcein and they are the bodies described by Badian as the chromosomes Badian stated that these structures took the Feulgen stain and this has been confirmed (Nagel and Carson, unpublished personal communication) Badian showed that these structures fuse end to end at copulation Harper proved that fusion of the nuclei in Phyllactinia is initiated by fusion of the centrioles, although Harper's techniques did not reveal any internal structure in the *Phyllactinia* centriole It seems probable that the rod-shaped bodies which Badian observed in fusion are components of the centriole, rather than chromosomes If the fusion of the nuclei in yeasts were initiated by end to end fusion of the centriolar bodies, the anomaly described by Badian in which a diploid chromosome is supposedly produced by the end to end fusion of two haploid chromosomes would be obviated However, this would mean that in yeasts the centriolar bodies are Feulgen-positive and stain with both aceto orcein and aceto-carmine while the chromosomes are Feulgen-negative, and do not stain with either aceto-orcein or aceto-carmine, but give a positive test for volutin

It may be difficult to accept the view that the conventional chromatin in yeasts is present in the centriole rather than in the chromosomes, while the

chromosomes are composed of volutin or metachromatin. However, Henneberg's description of the stages through which the volutin passes inside the vacuole corresponds rather well with the stages through which chromosomes normally pass, and Wager and Peniston's careful description of the threadlike

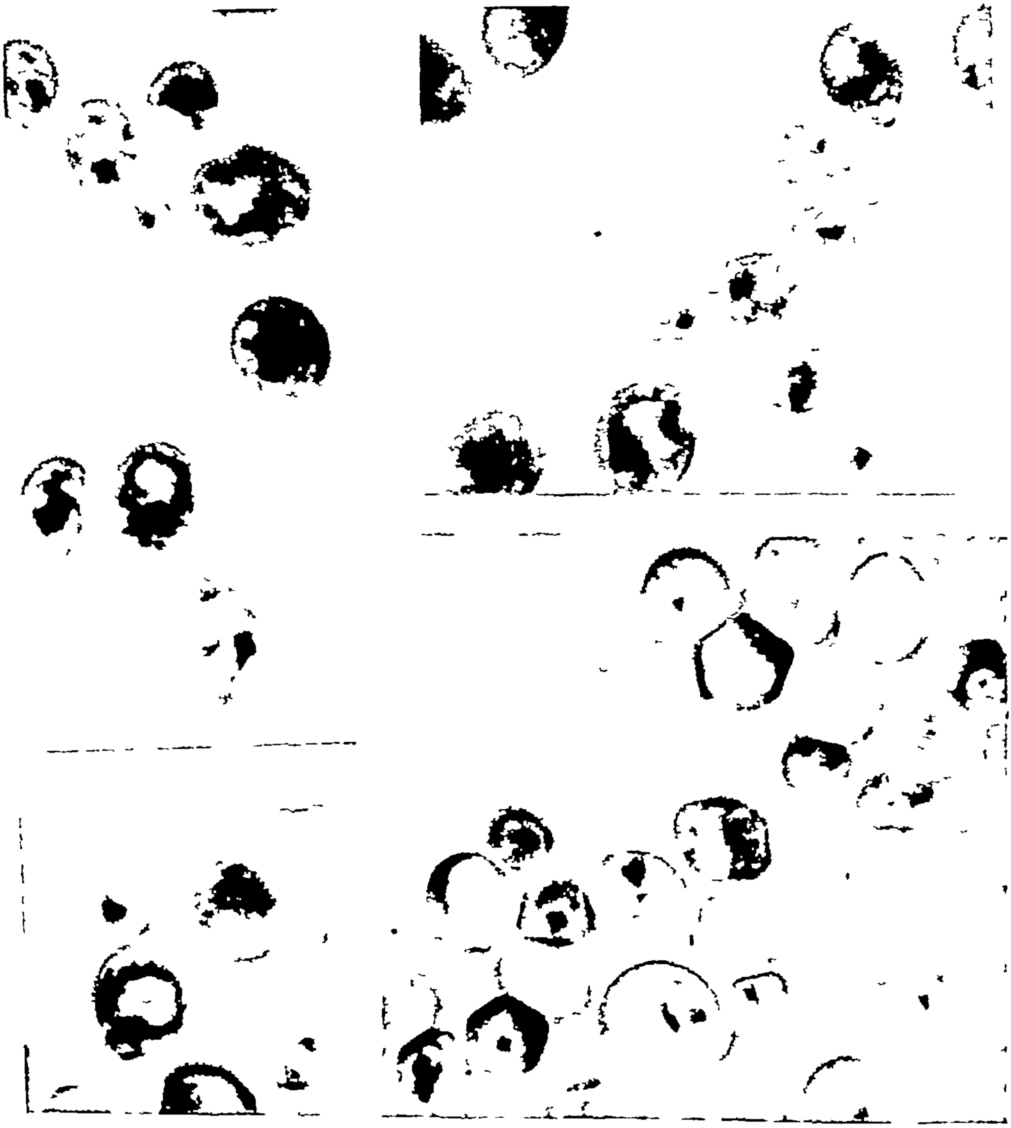

FIG. 10. Photographs of preparations stained with aceto orcein showing that two centriolar bodies are present in each cell, that they are not in the vacuole, and that they divide by pulling apart during the process of budding.

structures carrying chromomeres polarized to the centriole corresponds precisely with the modern concept of chromosomes and chromomeres.

We may conclude that the nucleus in yeasts is a compound structure containing the dense hemispherical centriole intimately attached to the nuclear

vacuole The vacuole is usually flattened on one side and otherwise is almost a perfect sphere The flattened side of the nucleus is the area of attachment to the centriole

When standard cytological techniques are applied to yeasts, a great deal of shrinkage and distortion of the yeast cell occurs, often accompanied by the disappearance of the vacuole It is only rarely that the vacuole remains intact when the cell is stained with aceto-orcein An effective technique for observing yeasts is to suspend the cells in water and allow water-soluble stains to diffuse slowly between the slide and cover slip The cells are observed as they take up the dye and before they become completely overstained This procedure does not involve so much shrinkage of the cell, which is an especially important consideration when one deals with small cells I have used this technique with 0 01 per cent methylene blue At the edge of the slide or on the border of a bubble, the chromosomes in living cells take on a deep blue color and appear as small, irregular, paired bodies usually free and in rapid Brownian movement *The important addition which this observation makes* to those of the earlier workers is that these bodies in the vacuole are paired This is an important common characteristic of chromosomes Methylene blue stain of the living chromosomes is evanescent, depending apparently on the oxidation potential within the cell Shortly after they take up stain the chromosomes disappear presumably because the oxidized dye is reduced to the leuco base This indicates that methylene blue passes through the reduced cytoplasm as the leuco-base and becomes oxidized on contact with the surface of the chromosomes The chromosomes tend to ball up into small, tightly-wound bodies that cease their Brownian movement and attach themselves to the inner face of the nuclear membrane I have observed the long threads retract toward a single point of attachment at the side of the nuclear vacuole The phenomenon has somewhat the appearance of a deliquescent crystal Eventually, one finds from one to six large, lenticular blue-black masses appressed to the inside of the nuclear vacuole Since the chromosomes are paired this represents the haploid number In old methylene blue or aniline blue lacto-phenol preparations, the cells contain from one to six clearly defined, blue bodies inside the nuclear vacuole produced by the attachment of the chromosomes to the wall of the vacuole These facts indicate that the dancing body, frequently described in the vacuole of the yeast cell, is composed of balled-up chromosomes In iodine-potassium iodide preparations, single spherical masses occur in the vacuole of almost every cell (fig 9) Under the influence of this fixative the chromosomes seem always to round up into a single dancing body Iodine-potassium iodide has the advantage of revealing the centriole with great clarity although it does not show the internal centriolar bodies

Toluidine blue used as a vital stain is the best stain that I have found for the chromosomes (figs 11 and 12) It does not seem to cause the chromosomes to "ball up" nearly so frequently as is the case with the other dyes and is not evanescent like methylene blue Usually about six bodies are observed in each vacuole In especially good preparations of diploids each of the six bodies

can be seen to be composed of a pair of chromosomes. In the germinating ascospore there are six single chromosomes. I have concluded that there are

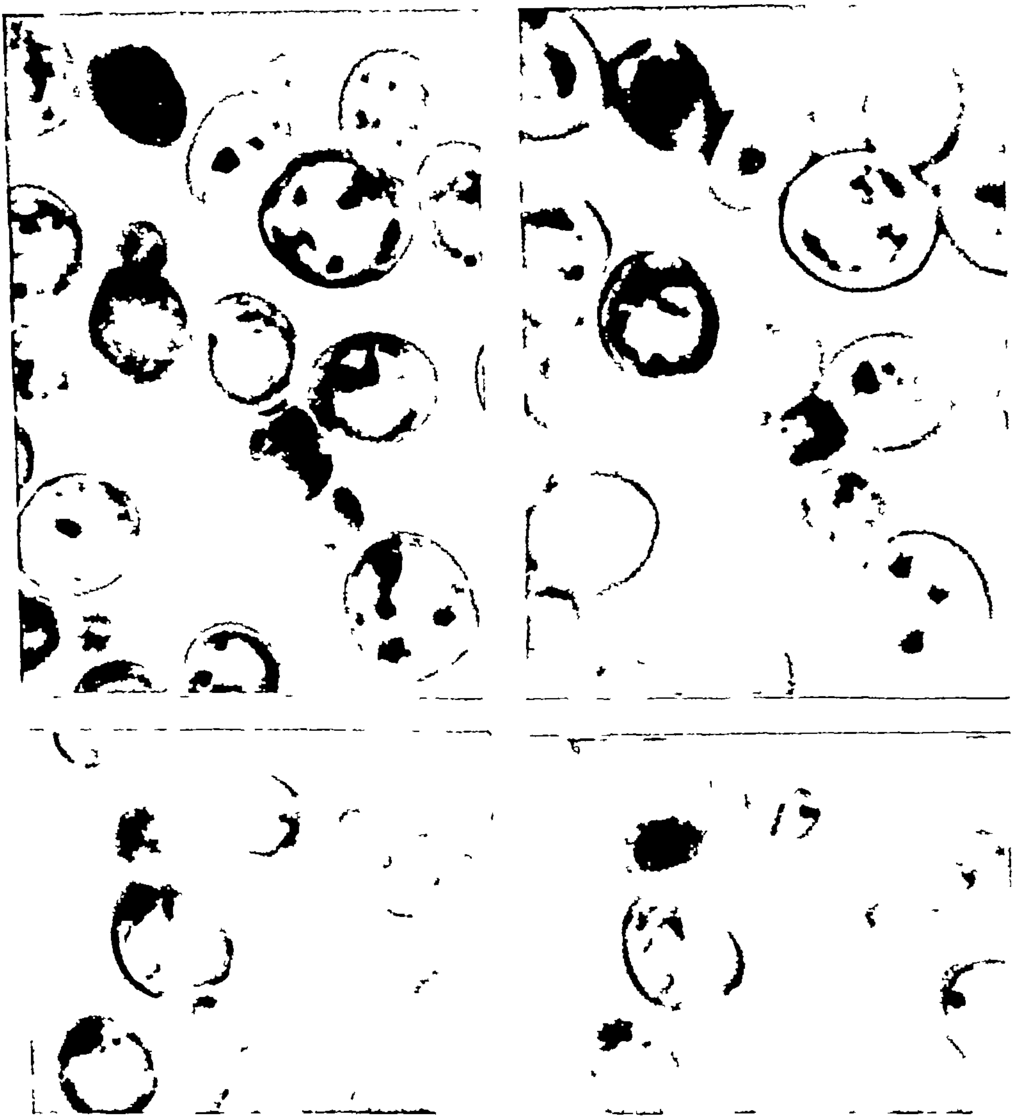

FIG. 11. Cells stained with toluidine blue showing the chromosomes in the vacuole. The two pictures in the upper row are two different optical levels of cells containing six large loosely contracted bodies inside the vacuole which stain with toluidine blue. It is not possible in this preparation to determine whether the bodies are paired. The pictures in the bottom row show a cell containing six pairs of highly contracted chromosomes (only four and a half pairs are in focus). The right hand picture shows three pairs which can be clearly differentiated and the left hand picture at a lower focus shows only single members of each of these three pairs of chromosomes.

twelve somatically paired Feulgen-negative chromosomes in the diploid cells of *S. cerevisiae* and that the haploid number is six.

Movement of the Chromosomes in the Living Cell

When a small drop of aniline blue in lacto-phenol is placed near the edge of a wet mount and allowed to diffuse between the slide and the cover slip, one can observe long, slender, delicately beaded, threadlike strands, vibrating in the nucleoplasm of some vacuoles These structures do not take the dye but seem merely to change their refractive index (possibly due to action of the acid or the phenol), so that they become observable Sometimes one larger, thicker strand, possibly produced by the coalescence of several strands, may be seen Even the slender strands seem to be relatively rigid, bending something like a very slender, but rather long, thin steel wire The chromosomes in this condition are only visible momentarily and soon disappear, but they resemble Wager and Peniston's figures closely enough to constitute confirmation of their observations

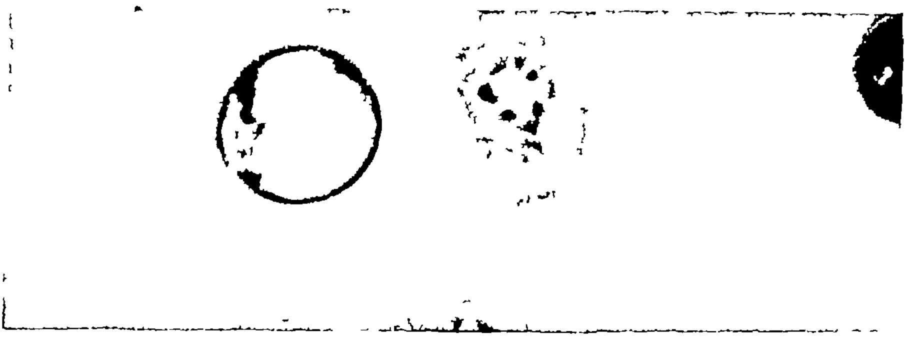

FIG 12 The central structure is a germinating four spored ascus One ascospore is in sharp focus and the wall of the spore can be seen enclosing the cell which consists of cyto plasm, crowded at one end, and the growing nuclear vacuole Six haploid chromosomes can be seen in the vacuole Parts of two other spores, one below and one to the lower left are visible, the fourth is not visible in the optical section The stain is toluidine blue

These observations indicate that the chromosomes in the living cell vibrate in the nuclear sap After one has observed the phenomenon in cells in which the refractive index of the chromosomes makes them visible, suggestions of the movement are visible in other yeasts such as *Torulopsis utilis*, *Saccharomycodes ludwigii*, and *Schizosaccharomyces octosporus* The motion may be visible in unstained material and may continue after flooding with Lugol's iodine solution which stains the glycogen brown and often brings the chromosomes into higher relief The vibration of the chromosomes should greatly facilitate the exchange of materials between the nuclear sap and the cytoplasm

VIII BUDDING

The ability of yeast cells to reproduce by budding has distinguished them from other fungi as well as from other organisms and the observations presented here show that the mechanism is quite unique The nuclear vacuole puts out a slender tube which forms and enters a small protuberance on the cell

wall (fig 13) The bud and an enlargement at the end of the vacuolar tube (the bud-vacuole) grow simultaneously

When a cell buds both the nuclear vacuole and the centriole divide The first step is the formation of the long, slender tube leading from the vacuole to the periphery of the cell This phenomenon can be observed only in cells containing enough glycogen so that the iodine stain delimits the vacuole and its tube as a clear space in the surrounding reddish brown cytoplasm Observation is facilitated by the use of a Wratten 45 filter which converts the reddish brown color of the cytoplasm to blue-black and reduces the chromatic aberration of the lens system The canal from the vacuole may originate any place on the surface of the vacuole, but usually appears at a point near the attachment of the vacuole and the centriole The bud is always produced near the centriole and when the canal emerges at the opposite side of the vacuole, the long, slender channel extends all the way from the most distant part of the cell through the cytoplasm and finally produces the bud near the centriole A bulb is produced at the end of this canal to form the bud-vacuole Occasionally, the opening between bud and mother cell is too small to permit the contents of

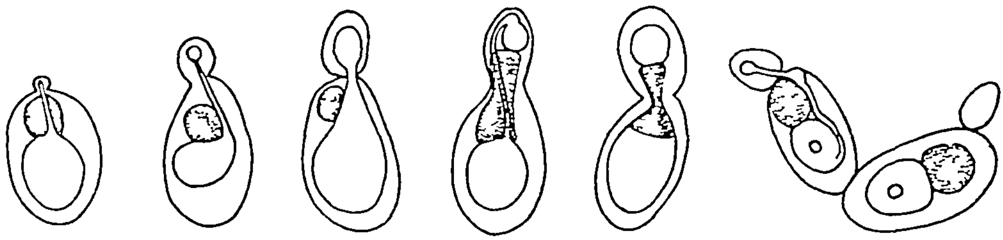

FIG 13 THE PROCESS OF BUDDING IN YEASTS DISCUSSED IN THE TEXT

the mother-vacuole to enter the bud-vacuole and the canal is distended at this point like the oesophagus of an ostrich swallowing an orange During this period the centriole is a hemispherical, solid, unyielding structure that is not deformed by movements of bodies near it After the bud-vacuole is formed, the centriole divides Sometimes in the Lugol's solution, it is seen as two bodies, each of which divides by stretching out and thinning out at the center After the division of the centriole is completed and the establishment of contact of bud-vacuole and bud-centriole has been attained, the interconnecting canal between the mother- and the bud-vacuole disappears

As soon as the bud approaches the size of the parent cell, the nuclear apparatus in the bud- and the mother-cell reorients itself so that the centriole in each cell is distal to the bud partition

IX STRUCTURE OF THE COLONY

Knowledge of life cycles and variations in bacteria has developed almost exclusively from the study of isolated individual cells, without consideration of the structural organization of the colony Legroux and Magrou (27) studied the structure of colonies of *Vibrio cholerae* and of a number of other bacteria

They discovered that rod-shaped variants of Vibrio appear in perpendicularly arranged packets on the exterior of the colonies, held together by a transparent substance apparently exuded from the cells They assumed that this substance gave the rough colonies their rigidity The rod-shaped organisms in the outer layer of the colony were made up of a central region and an exterior The central region underwent division into two or four rounded particles, often of unequal size This phenomenon was found in Vibrio, in the typhoid bacillus, in the diphtheria bacillus, and in the tubercle bacillus

Pisova (59) found that, when a yeast colony grows on agar, a pseudo mycelial growth of long fibrous cells penetrates the agar, especially at a high sugar concentration After a few days or weeks, the surface cells begin to autolyze, continuing until an outer layer of autolyzed cells is formed Lindegren and Hamilton (41) repeated Pisova's work After yeast colonies had been grown on malt-yeast agar, portions of the agar, containing colonies, were cut out and dropped into Flemming's solution The material was imbedded in paraffin Sections were made and stained with a variety of dyes Direct smears were also made by cutting the fresh colony in half vertically with a razor and pressing the exposed section gently against the slide

In a section of the yeast colony, the outer layer of autolyzed cells stains very lightly, and the inner central mass of vegetative cells with their dense protoplasts is much darker A pseudo-mycelium of yeast cells penetrates the agar and is thickest and deepest at the edges of the colony, apparently where oxygen is most abundant The thin peripheral film of cells at the edge of the colony spreads over the surface of the agar At the points of origin of penetration of the agar the growth of cells is very abundant This may be due to the channeling of the substrate nutrient into these regions along the cracks made by the pseudo-mycelium The central vegetative cells are extremely small, indicating that cell division continued after the nutrients became less readily available and competition resulted in a decrease in cell size A few cells in the outer autolyzed layer produce asci Autolysis apparently occurs early in the history of the colony, at least before competition reduces cell size The autolyzed layer contains the only asci found, suggesting that autolysis supplies essential nutrients on which sporulation depends

In some of the contact smears the autolyzed cells were not so shrunken as those obtained by the paraffin method The walls seemed relatively intact, but there were no stainable cell contents The "ghost" cells were larger than the densely stained cells in the vegetative section of the colony These autolyzed cells, which apparently serve as sources of nutrients for the sporogenous cells, have a parallel in the paraphyses found in pyrenomycetes and discomycetes which also act as nurse cells In some regions of the autolyzed layer, small clusters of round, apparently haploid, cells were found, suggesting that some spores may germinate in the layer If copulations occur this could be a source of recombinations producing new genotypes

The striking parallelism between the structure of yeast colonies and those of bacteria, as shown by Legroux and Magrou, suggests that the life-cycles may be

similarly parallel. Yeasts possess a nuclear mechanism, and the vegetative cells undergo meiosis and sporulation in the outer layer of the colony. In bacterial colonies a similar division of the cell contents into two or four bodies occurs in the corresponding layer. The analogy suggests strongly that the granulation of the bacterial cell is the result of a reduction division similar to that known to occur in yeasts. The bacterial cells in the outer layer of the colonies may be homologous to the ascospores of yeasts.

Harper (21) pointed out that the ascomycetes are differentiated from all other living organisms by the capacity for "free cell" formation. This is a unique type of spore formation in which the spore is cut out of the cytoplasm by the astral rays originating from the centriole. Usually four or eight spores are produced in a single ascus with the production of a certain amount of residual cytoplasm called the epiplasm. In all other types of cell formation, cells are usually cut out of a syncytium by cleavage without residual cytoplasm. In the Bacillaceae, the spores are formed as free cells and an epiplasm is produced. Since this characteristic distinguishes the ascomycetes from all other living forms, this fact automatically includes the Bacillaceae in the ascomycetes.

The Coccaceae, however, are distinct from the Bacillaceae for I have shown that in *Micrococcus ochraceus* (37) the autogamous copulation is followed by a reduction division in which the spore mother cell produces the tetrad by cleavage, rather than by free cell formation. In this form the chromosomes were demonstrated as strings of chromomeres (gene-strings) which synapsed chromomere to chromomere and finally underwent reduction after forming a typical reticulate nucleus.

X. DORMANCY

Diploid cells which have been grown on pre-sporulation agar for a week or more (but have not yet sporulated) become filled with stored fat and carbohydrate and, as a result of these reserve accumulations, become dormant. When tested in a Warburg apparatus, they are unable to give off CO_2 or consume O_2. Accessory substances may be involved in the induction of dormancy. Dormancy can only be broken by presenting the cells with a nutrient containing sufficient vitamins, carbohydrate, and a nitrogen source to insure continued growth. Vegetative cells which have grown on rich natural substrates, such as ripened fruits, fill with reserves and are probably dormant. They may germinate from dormancy by a simple vegetative procedure, or the diploid dormant cells may sporulate when placed on gypsum. In the latter case, conditions are unfavorable for continued vegetative growth just as they would be in a sandy soil. These observations suggest that the vegetative cells grown on ripe fruit become loaded with reserves and turn dormant. If they fall on another fruit or into a rich sugary nutrient, they germinate and grow vegetatively. At the end of the season when the fruits finally fall on the soil, sporulation occurs and the spores germinate the following spring.

It has long been known that yeasts store both fats and carbohydrates and the principal conditions controlling the storage of these reserve materials have been

fairly well worked out It was not known, however, that cells containing abundant accumulations of reserve materials are in a state of dormancy, they are unable to take up O_2, to give off CO_2, or to bud, either in a phosphate buffer solution or in a buffer-glucose solution in the Warburg apparatus, but they begin to grow when they are brought into a complete nutrient medium The mechanism has a high survival value, since it prevents cells from "wasting" their reserves, since growth can only begin under conditions in which continued or considerable growth is possible

Meissner (56) studied the appearance and disappearance of glycogen in the yeast cell and showed that cells filled with glycogen produce more than the theoretical amount of CO_2 in fermenting a sugar substrate He also found that glycogen accumulates in the cell and attains a maximum at the end of the principal fermentation when it begins to disappear from the cell, even before all the sugar is consumed He designated glycogen as a temporary reserve used by the cell through an endogenous diastatic enzyme He pointed out that the deposition of an insoluble carbohydrate inside a semi-permeable membrane enables the cell to take in soluble carbohydrate continuously by osmosis

Wager and Peniston (74) studied the same question with cytological techniques They found that glycogen was deposited in the cell in the form of small granules which coalesced to form a solid mass of glycogen almost completely filling the cell

McAnally and Smedley-Maclean (53, 54, 55) and Smedley-Maclean and Hoffert (63, 64) showed that both carbohydrates and fats accumulated in the cell as a result of continued feeding with sugars They found that phosphates increased the deposition of both reserves and that maltose seemed to increase the carbohydrate reserve In addition, they pointed out that an excess of oxygen favored storage of fat

Henneberg (22) also pointed out that the presence of phosphates favored the deposition of glycogen, while chlorides seemed to inhibit its deposition He found that the maximal protein content of the cell was 67 per cent but that yeasts containing stored reserves might contain only 22 per cent protein He stated that the amount of protein in a cell stands in inverse relation to the amount of glycogen (and presumably fat) He pointed out that the yeast cells collected directly from fruits are generally rich in glycogen and furthermore that yeasts kept in moist condition on filter paper live longer if they contain large amounts of glycogen Some apiculate and lactose fermenting yeasts were unable to store glycogen

Lindegren (39) observed that the vegetative budding yeast cell, in the logarithmic growth phase (fig 14*a*), which contains a very large centrally located vacuole and stains a light golden yellow with iodine, contains a few tiny fat globules If these cells are placed under conditions of relatively low oxygen tension and supplied with an abundance of sugar, after budding has ceased, granular deposits of glycogen appear in the cytoplasm, which finally deform the vacuole and diminish its size (fig 14*g*, *h*) If the cells are well aerated and supplied with an abundance of sugar, fat globules appear and increase in size

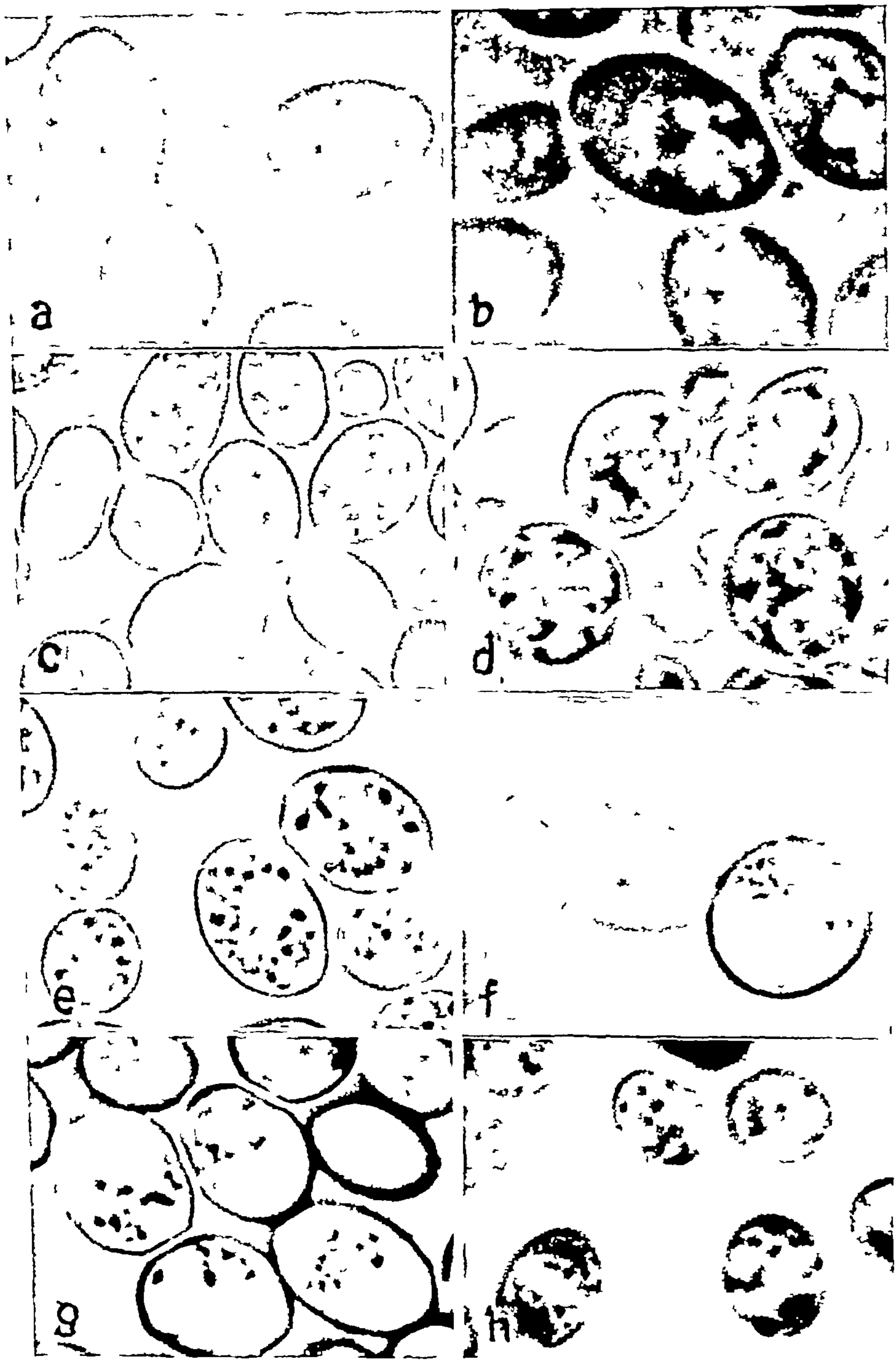

FIG 14 *a* Budding vegetative yeast cells at the logarithmic stage of growth showing a single vacuole characteristic of this condition

b Budding yeast cell in the lag phase showing the apparently multiple vacuole resulting from deformations of the single vacuole by interference of reserve material

c Dormant vegetative yeast cells loaded with fat and glycogen, grown on pre sporulation agar the dark color is glycogen stained with iodine

d Germinating dormant cells from pre sporulation agar, showing the vacuole breaking through the enclosing net work of fat and glycogen

e Cells loaded with fat by growth in aerated sugar solution

f Cell of the type shown in *e* germinating

g Cell grown in sugar under conditions of reduced oxygen tension loaded with glycogen, a few fat globules surrounding vacuole

h Cell of the type shown in *g* stained with iodine, revealing the deformation of the vacuole by the reserve materials

and number and also tend finally to obscure the vacuole, although it retains its spherical form (fig 14e)

Granular Glycogen

The granular carbohydrate reserve in yeasts stains dark reddish brown with Lugol's iodine-potassium iodide solution In many cells one can observe twenty or more small isolated granules Occasionally, these granules are linked by connecting bands Hundreds of small glycogen granules can be seen occasionally in some cells in addition to fifteen or twenty larger ones These observations suggest that, in most of the cells containing a solid mass of dark-staining glycogen, the distribution is similarly non-homogeneous with the basic granular structure obscured by overstaining

Unstained cells containing glycogen can be recognized by the high refractive index of the cytoplasm (fig 14g) The nuclear vacuole in a glycogen-containing cell is often concealed by the glycogen The visible vacuoles often appear to be multiple, but critical observation shows that the small vacuoles are all interconnected with each other by fine canals and are merely separate compartments of one major vacuole This is consistent with the view that the vacuole is the nucleus and that each yeast cell contains only a single vacuole In many unstained glycogen-containing cells no vacuole is visible, but staining with Lugol's solution always reveals the vacuole either compressed into the middle of the cell by a surrounding sheath of glycogen, or at one pole of the cell (fig 14h) The vacuole in a glycogen-containing cell is usually much reduced in size

Budding is retarded or inhibited in cells containing much glycogen, and occurs only after the glycogen has begun to disappear from the cell Growing cells during the lag phase contain enough unidentified reserve to obscure or deform the vacuole (fig 14b) After the cell has completed one or two divisions, the refractive index drops and the vacuole reappears or loses its deformity

Deposition of granular glycogen is irregular at high (12 per cent) concentrations of sugar and rarely fills the whole cell The stained granules are darker than those observed at lower concentrations of sugar Many small granules may coalesce, often forming two large polar deposits Small granules of glycogen may be linked by arcs of glycogen At lower sugar concentrations (4 per cent), the deposition of glycogen continues until it fills the entire cell with the exception of a small region at the one end into which the vacuole is crowded, or the vacuole may be concealed in the center of the cell inside the spherical envelope of glycogen After the deposit has reached a maximum, the glycogen disappears on aeration, by peripheral disintegration or solution, with a decrease in the density of the glycogen mass Finally a single large diffuse granule is found in the cell, and the vacuole has regained its original size Deposition of glycogen does not ordinarily begin until at least half the total number of cells that are to be formed are present Under favorable conditions, involving low oxygen tension and continued addition of sugar, all the cells may become filled with glycogen

Stainable glycogen does not accumulate in well-aerated yeast cultures grown in 1 per cent glucose broth The nuclear vacuole attains its maximal size under these conditions

Glycogen is never found in the vacuole of living cells, but some dead cells contain glycogen in the vacuole Most of the dead cells remaining in a culture which has been reactivated after glycogen deposition contain glycogen in the cytoplasm Either glycogen tends to deposit in dead cells or dead cells are unable to metabolize their accumulation of glycogen

Non-Granular Glycogen

Dark brown (granular) glycogen is deposited rather regularly in deep broth cultures in test tubes Glycogen-free cells (which usually contain about 20 per cent of an unspecified carbohydrate) grown in aerated 1 per cent glucose broth stain golden yellow with iodine Cells from well aerated cultures which have received additional sugar differ from both the above by staining with iodine without any dark brown granular deposit The entire cytoplasm is light brown, suggesting general distribution of a non-granular carbohydrate throughout the cell The vacuoles are round, centrally located and usually large, indicating that the non-granular carbohydrate does not deform the vacuole, at least in the early stages of its deposition

Fat

Fat is also stored in yeast, if the culture is well aerated and well supplied with phosphate and sugar Fat first appears as an accumulation of highly refractive droplets around the vacuole Deposits are often polar or comprise a network of streptococcus-like threads of granules which sometimes branch and are closely appressed to the outer surface of the vacuole In most types of *S cerevisiae* the fat globules tend to increase in numbers and to enlarge individually as the culture becomes older and storage increases The addition of alcohol or the application of heat causes many of the fat globules to coalesce Dead cells usually contain coalesced fat globules, just as they often contain glycogen Fat invariably accumulates in yeast cells in shallow Erlenmeyer cultures

Dormant Vegetative Cells

After most yeasts have grown for several weeks on slants of our pre-sporulation agar, the cells contain abundant deposits of fat and glycogen, and although all the cells are alive, they are dormant (fig 14*c*) Subsequent experiments were undertaken to reproduce dormancy by loading the cells with reserve materials in broth cultures, but we were unable to obtain cells with precisely the appearance of those taken from pre-sporulation agar, which appear to be much more heavily packed with larger fat granules The vacuoles in a fat-filled cell from pre-sporulation agar are usually spherical, indicating that fat and glycogen deposition on this medium occur without deformation of the vacuole After an hour in nutrient medium, much of the fat disappears from most of the

cells and after the second hour, half of the cells show buds and some contain a few fat globules The vacuoles appear to be multiple or obscured in the growing cells (fig 14*d*) In three hours, there is very little visible fat left in the cells, nearly all of which contains multiple or obscured vacuoles Practically all the cells bud, showing that the culture is viable

Manometric studies were made with the cells taken directly from the pre sporulation slant (fig 14*c*) and shaken with phosphate buffer containing 4 per cent glucose, but with no other nutrients The Q values showed that the cells were incapable of taking up oxygen and incapable of producing CO_2 either aerobically or anaerobically over a 150-minute period During the same period, all the cells in the nutrient broth budded Ninety-eight per cent of the cells from the Warburg vessel showed little or no change

Cells of a standard baking yeast, strain A, which had been grown on pre-sporulation agar, were collected from the agar surface and washed with M/15 KH_2PO_4 Three Warburg vessels were inoculated with equal amounts of dormant cells Each vessel contained 4 per cent of glucose in solution The first received phosphate buffer, the second received 1 per cent of corn-steep-water solids, and the third received 0 3 per cent ammonium sulfate, biotin (2 γ per liter) and pantothenic acid (200 γ per liter)

Since our culture of *S cerevisiae* is incapable of synthesizing biotin and panto thenic acid, these substances were added, together with ammonia, to see if they would break the dormancy of the fat-filled yeast cells The cells suspended in sugar were dormant and gave off no CO_2 after over five hours in the Warburg apparatus, but the cultures in the other vessels fermented the sugar, the action being much more rapid in the richer nutrients This experiment cannot always be duplicated, because cells from the pre-sporulation agar slants are not always in precisely the same condition, since some cultures sporulate directly in the slant

Storage of Fat and Carbohydrate

Storage of reserves only occurs in a medium in which growth has nearly ceased A 1 per cent glucose broth was prepared with half the standard amount of nutrient broth to insure the early cessation of growth Fifty ml of broth in 500 ml Erlenmeyer flasks were inoculated and shaken for 48 hours The suspension of cells from some of the Erlenmeyer flasks was placed in 8 x 1 inch tubes to favor the development of glycogen, while the remainder were kept on the shaker to favor the development of fat Tests with Fehling's solution were made to determine when the sugar disappeared, and sugar was added as soon as a deficiency was indicated Phosphate was also added, since this is known to increase the deposition of both fat and glycogen The addition of sugar was continued for four days

Three cultures were used, two standard baking yeasts, strains L and R (*S cerevisiae*) and a hybrid (*S cerevisiae* X *S globosus*) Strain U stored both fat and carbohydrate relatively uniformly, as indicated by microscopic ex-

amination Strain R stored carbohydrate well, but the accumulations of fat were irregular and the fat-containing culture was discarded The hybrid stored fat in large clusters of extremely tiny granules approaching the limits of visibility The cells appeared to be crowded with stored materials, but chemical analysis (ether extraction) revealed that only 6 16 per cent fat was present The hybrid stored carbohydrate poorly Nitrogen analyses were converted to protein by multiplying by the factor 6 25 (table 1) The sample was ashed, and the difference was calculated as carbohydrate

Yeast cells may contain different amounts of carbohydrate and fat reserves These reserves hinder the respiratory, fermentative, and budding activity of the cell The Q values are reduced nearly to zero After a lag, the dormant cells begin to respire, ferment, and bud The low Q_{O_2} values of the nearly dormant cells are due to causes different from those responsible for the low

TABLE 1

Chemical analyses of cells containing visible deposits of fat and glycogen compared to normal cells

CULTURE	VISIBLE DEPOSIT	PROTEIN	FAT	ASH	CARBOHYDRATE
U	None	61 3	4 83	8 48	25 4
	Fat	25 0	31 25	5 22	38 5
	Glycogen	31 9	16 96	5 33	45 7
CxGII	None	54 4	17	9 29	36 1
	Fat	39 4	6 16	7 09	47 3
R	None	53 8	7 47	9 15	29 5
	Glycogen	31 9	5 24	5 22	57 7

values of cultures containing large numbers of dead cells In the early phases of this work, cells were grown in 8 per cent sugar, peptone, yeast-extract medium and in this medium, 50 to 90 per cent of the cells died, especially if the cultures were well-aerated The dead cells gave normal $Q^{O}_{CO_2}$ and $Q^{N}_{CO_2}$ and values ranging from 300 to 450, but with Q_{O_2} values of 0 The dead cells were unable to consume O_2, although they were able to ferment Some of our dormant cultures contained nearly 100 per cent viable dormant cells These viable dormant cells, loaded with reserve materials, were unable to consume O_2 or to evolve CO_2, but this situation is obviously different from that found when the cultures contain many dead cells

The reproducibility of results obtained by the Warburg respirometer depends upon the absence of accumulations of reserve materials in the cells Cells should be aerated and transferred to the Warburg apparatus when a minimum number of dead cells is present and a considerable proportion is actually budding In order to make reproducible analyses, growth must be stopped in an active phase and the cells washed with M/15 phosphate If the cells are al-

lowed to stand in a nutrient medium, they will accumulate some reserve fat or carbohydrate, or both, depending on the aeration and the concentration of sugar

Accumulated reserves are responsible for the lag in growth observed on the inoculation of the fresh medium The lag can be completely eliminated if the cells are transferred before any storage has occurred

Hansen (20) developed a technique of preserving yeast cultures by growing them in 10 per cent sugar broth and allowing the culture to dry down The excess of carbohydrate caused growth to cease and storage of reserves brought the cell into dormancy This procedure could not be used to supply dormant cells in our experiments because a very large percentage of the cells die in high concentrations of sugar and only a few attain full dormancy Winge and Hjort (77) recovered living cells from fifty-year-old cultures prepared by Hansen

The fact that dormant cells require a medium containing a relatively full complement of nutrients enables the cells to start growing under conditions that assure continued growth The fact that they require specific vitamins which they are unable to synthesize may have some importance in solving the problem of inducing other fungal spores to germinate

XI VITAMIN SYNTHESIS

A survey by Burkholder, McVeigh, and Moyer (8) has revealed that practically all yeasts can synthesize riboflavin, but that they vary considerably in their ability or inability to synthesize the other B vitamins Our cultures of *S cerevisiae*, *S carlsbergensis*, *S globosus*, and *S bayanus* differed from each other in the ability to grow on media deficient in different B vitamins A study of eight bakers' yeasts collected on the market showed that all these strains of *S cerevisiae* resembled each other rather closely in their vitamin requirements, even on a quantitative basis They were all unable to synthesize biotin Growth in the medium without pantothenic acid was always less than with all the vitamins, and the only considerable variation encountered was in response to the absence of this vitamin Some yeasts did slightly better when niacin was omitted from the medium than when it was present, and all grew nearly as well in the absence of inositol, thiamine, and pyridoxine as they did when all six vitamins were present Burkholder (6) has shown that other strains of the *S cerevisiae* do not conform to these bakers' yeasts in their ability to grow in the absence of specific B vitamins This suggests that the close similarity of the eight bakers' yeasts may indicate that they are all closely related

Our culture of *S carlsbergensis* differed from the baking yeasts in being able to synthesize biotin, but unable to synthesize pyridoxine The technique was that developed by Burkholder and Moyer (7) The standard medium containing glucose and asparagine and various minerals was supplemented with the six B vitamins (exclusive of B_2) In the medium containing the six vitamins, growth was nearly complete at the end of three days Other nutrient media were made up corresponding to the complete medium described above except

that single B vitamins were lacking. Since these B vitamins are essential to cell metabolism, it is assumed that a culture able to produce good growth in a nutrient lacking a given vitamin is able to synthesize this vitamin and that the converse is also true. The amount of inoculum was tested and shown not to carry enough vitamin to obscure the results.

A hybrid was made between *S. carlsbergensis* and one of the baking yeasts, *S. cerevisiae*. The data on the pedigree are recorded in table 2. The turbidity readings of yeast growth in tubes from which pyridoxine, pantothenic acid, and biotin respectively, are absent, but the remaining five vitamins present

TABLE 2

Turbidity readings of yeast grown in culture media lacking pyridoxine (Py.), or pantothenic acid (Pa.), or biotin (Bi.). The cultures used are from a pedigree of S. carlsbergensis by S. cerevisiae

GENOTYPE	DIPLOID			ASCUS NO.	ASCOSPORES											
					A			B			C			D		
	Py.	Pa.	Bi.		Py.	Pa.	Bi.	Py.	Pa.	Bi.	Py.	Pa.	Bi.	Py.	Pa.	Bi.
S. carlsbergensis	24	280	280		22	200	125		d*			d			d	
S. cerevisiae	350	220	11			d		350	15	12	310	20	8		d	
(S. carls. × S. cer.) A × B	315	240	135													
(22 200 125 × 350 15 12)				1	274	45	50	312	202	55	290	200	59	300	210	110
				2	355	293	170	80	65	160	350	220	75	25	15	7
				3	318	212	140	100	210	140	300	235	134	345	235	110
1A × *S. cerevisiae* B (274 45 50 × 350 15 12)				4	310	25	145	325	28	145		d			d	
				5	290	13	10	4	5	4	378	25	45	295	4	4
1D × *S. cerevisiae* C (300 210 110 × 310 20 8)				6	340	237	275	93	15	—	358	16	230		d	
				7	345	135	24	278	14	67	350	50	10	420	22	60
1A × 1D (274 45 50 × 300 210 110)				8	318	256	160	360	22	193	340	240	200	320	—	170

* d = died

are shown. A high reading indicated a large number of cells while a low reading indicated that few cells are present in the suspension. The diploid culture of *S. carlsbergensis* failed to grow in the absence of pyridoxine, but grew in the absence of both pantothenic acid and biotin, and the single surviving haploid ascospore culture likewise failed to grow in the absence of pyridoxine. The diploid culture of *S. cerevisiae* grew in the absence of both pyridoxine and pantothenic acid, but failed to grow in the absence of biotin. Two surviving ascospores which were tested, grew in the absence of pyridoxine but were unable to grow in the absence of either pantothenic acid or biotin, suggesting that the strain may be heterozygous for a gene pair controlling the synthesis of pantothenic acid. An interspecific hybrid between a pair of *S. cerevisiae* and *S.*

carlsbergensis haplophase cultures grew well in the absence of all three vitamins The twelve ascospore cultures from three asci of the interspecific hybrid were tested for their ability to grow in the absence of the three vitamins In spite of the fact that one parent produced a growth of 22 and the other 350, in the absence of pyridoxine, nine of the haplophase progeny from the hybrid produced about 300 units of growth Two were better than 80 and only one resembled its *S carlsbergensis* parent in being unable to grow well in the absence of pyridoxine, but it grew so poorly in the absence of both pantothenic acid and biotin that its poor growth probably resulted from some other more fundamental deficiency The fact that the first generation segregants from the hybrid all grew fairly well in the absence of pyridoxine suggests that some cytoplasmic effect may be obscuring any gene-segregations that may have occurred In the absence of pantothenic acid also the growth of these haplophase cultures resembled the better growing rather than the weaker parent Only one of the twelve gave the same poor growth in the absence of pantothenic acid as its *S cerevisiae* parent and this yeast was apparently weak in other respects as well In the absence of biotin, the same absence or obscuring of the Mendelian ratio occurred The progenies of these three matings gave irregular results suggesting that a cytoplasmic mechanism may be obscuring the Mendelian ratios The characters may be fundamentally under genetic control, but the ratios may be obscured by cytoplasmic effects If many genes were involved, rather than cytoplasmic obscuring of the Mendelian ratio, a greater variety of progeny would be expected This pedigree resembles that of *S bayanus* × *S cerevisiae* in which the galactose-fermenting *S cerevisiae* transmitted the capacity to ferment galactose to all the progeny in the pedigree (Lindegren, 34) In the backcross in which both parents were incapable of synthesizing either pantothenic acid or biotin, the fact that the progeny were generally inferior suggests that adaptations do not seriously complicate the interpretation Leonian and Lilly (28, 29, 30) derived yeasts capable of synthesizing vitamins from cultures incapable of performing the syntheses Their procedure probably favored the selection of mutations In our experiments, we attempted to minimize this possibility Interspecific crosses such as these are generally quite complex because the two genomes usually have a greatly different collection of modifiers and the expression of each gene is influenced differently than it was in the original environment

S globosus is capable of synthesizing pantothenic acid, but incapable of synthesizing thiamine A hybrid was made between a haplophase from a homozygous pantothenic-deficient culture of *S cerevisiae* (different from the one used above) and a haplophase culture of *S globosus* Several hybrids were produced by this mating, and one of them sporulated well, but only a few of the ascospores were viable One of the hybrid-haplophases was backcrossed to the original pantothenic-deficient *S cerevisiae* The resulting diploid synthesized both pantothenic acid and thiamine efficiently Since neither parent could synthesize both vitamins, the hybrid had obviously obtained its ability

to synthesize pantothenic acid from *S globosus* and thiamine from *S cerevisiae* The hybrid was a poor synthesizer of biotin, but this was according to expectation, since neither parent possessed the ability

XII ADAPTATION

The ability of an organism to adapt itself to the environment depends upon its capacity for producing variants Variation in yeasts depends upon both genetical and cytoplasmic mechanisms The genetical mechanism produces variations through *a*, the operation of segregating the chromosomes together with the aberrations of this mechanism, *b*, by mutation, especially in the haplophase, and *c*, by matings which produce new combinations of different genotypes to form new diplophases

Plastogenes, Plasmagenes, and Cytogenes

The cytoplasm is also a possible source of variation Darlington (9) has given names to two kinds of cytoplasmic components which are found in higher plants the plastogenes and the plasmagenes The plastogenes are contained in plastids similar to the chloroplasts that have long been recognized as self-perpetuating cytoplasmic bodies which may vary and produce changes in the organism either independently or under the influence of the genes of their host (Rhoades, 61) Winge and Laustsen demonstrated that there are certain cytological constituents, which they assumed to be the chondriosomes, that divide regularly at each nuclear division and which may produce degeneration if they diminish in number It is possible that Darlington would include the cytoplasmic components described by Winge and Laustsen under the category of plastogenes Chloroplasts are typical plastogenes but they do not divide regularly at each cell division, for a deficiency in chloroplasts can be made up by a cell which has received less than a standard number simply by multiplication of the chloroplasts while the cell is in the resting condition If we include the cytoplasmic components described by Winge and Laustsen under the heading of plastogenes, we recognize two categories of these organelles, one which multiplies independently of nuclear division, and the other which divides regularly at each nuclear division

Plasmagenes, in Darlington's view, are related to viruses inasmuch as they are relatively independent of any definitely recognized plastids or cytoplasmic organelles, but apparently multiply more or less independently in the cytoplasm He considers them to be the phylogenetical forerunners of viruses I (32) have developed a hypothesis suggesting that viruses were developed from the genes of the host by partial digestion and mutation in insect vectors

The cytogene (Lindegren, 34) is a third type of cytoplasmic component capable of producing variation and in effecting the adaptation of yeasts to different substrates The cytogene is a self-perpetuating entity, initiated by the action of a gene but made specific by a specific substrate under whose influence it becomes self-perpetuating (Spiegelman, Lindegren and Lindegren, 71)

Spontaneous and Induced Mutations (?)

In the adaptation of microörganisms to a specific substrate, while they are increasing in numbers on that specific substrate, the question arises as to whether the substrate induces the change resulting in adaptation, or whether the change occurs spontaneously and the substrate acts merely to select the new variant, permitting it to outgrow the original normal form There are, therefore, two fundamental hypotheses *a*, that the microörganisms may produce a new variant spontaneously, independently of the substrate, and *b*, that the contact of the substrate with the microörganism induces the variation, which is transmissible to the progeny of the microörganism

Previous to the analysis of this problem by Luria and Delbrück (52) experienced workers could not determine with the available data which mechanism functioned For example, when *Bacterium coli mutabile* (a non-lactose fermenting organism) is plated on lactose agar, papilli appear on the colonies at the point of origin of lactose fermenting variants This phenomenon was studied intensively by Lewis (31) and Stewart (72), who obtained substantially the same results, but drew precisely opposite conclusions Lewis concluded that the variation from non-lactose fermenter to lactose fermenter is independent of the substrate and occurs spontaneously in all cultures whether or not lactose is present, while Stewart concluded that transformation from non-lactose fermenter to lactose fermenter is induced by lactose Luria and Delbrück have shown that the question can not only be answered definitively, but that the rate at which new forms are produced can be precisely calculated However, a more subtle mathematical approach is required than previous workers had used (I am indebted to Dr A D Hershey for explaining to me the significance of Delbrück's mathematical analysis) They analyzed the question in which the *transformation of bacteria from virus sensitivity to virus resistance* was involved The same approach can solve the question of the effect of lithium chloride, antisera, bactericidal substances, or specific substrates such as the different sugars, on the selection or induction of variations in multiplying cultures The procedure is as follows A number of culture tubes without the specific bactericidal substance (or the virus, or the carbohydrate substrate) are all inoculated simultaneously and allowed to grow, then finally sampled and tested for the normal form and the adapted variant The conditions are that *a*, the normal and the variant forms grow about equally well in this initial non-selective nutrient medium and *b*, that the probability of the variation occurring is relatively small If the variation is not induced by the substrate, adapted forms may appear either early or late as the culture grows *in* the absence of the substrate Random spontaneous production of the adapted forms without competition will result in a great variation in the number of adapted forms present in the different tubes Large variations in the number of adapted forms appearing when samples of organisms (grown in the absence of the specific inhibitor or substrate) are planted on the specific inhibitor or substrate, prove that the new variant appeared independently of the environmental condition to which it is adapted. Furthermore, the specific frequency of mutation can be

accurately calculated Luria and Delbrück point out that their use of the term "mutation" is merely formal On the basis of their analysis, Demerec (11) was able to show that resistance of *Staphylococcus aureus* to penicillin was the result of a number of heritable changes and that these changes were not induced by the action of penicillin, but originated spontaneously Since this type of analysis has not yet been made in the case of *B coli mutabile*, it is not possible to say whether this variation is spontaneous or induced At the present time, only the two cases, *a*, resistance to penicillin by *Staphylococcus aureus* and *b*, resistance to bacteriophage of the colon bacillus have been analyzed and both have been found to occur spontaneously, independently of the environmental agent Variation in bacteria involving changes in colony morphology are not generally referable to any substrate, although they have been correlated with transformation from virus-susceptible to virus-resistant forms, but Luria and Delbrück point out that a difficulty in analyzing this phenomenon arises from the fact that an entire colony is necessary to express the character There is no evidence, however, that colonial characteristics can be induced by an interaction between cell and substrate, on the contrary, there is every indication that this is a character which arises spontaneously Since often no environmental effect seems to be involved, the difficulty of analysis existing in the case of adaptation does not apply

Werner in a forthcoming article points out that the rate of mutation may not be so precisely calculable as Luria and Delbrück's article suggests unless more precise data on growth rates are available The mutant probably only rarely grows at precisely the same rate as the parent form, and Werner has discovered that small differences in growth rates must be considered in calculating the mutation rate

Induced Cytoplasmic Adaptation in Yeasts

We were able to demonstrate the existence of an induced adaptation in yeasts in the study of an illegitimate diploid (Lindegren, 34) The culture sporulated only rarely and did not sporulate at all in broth This fact eliminated genetical variation except for the unusual dominant mutations When a transfer was made from malt extract agar to corn-steep-water agar, only small numbers of the cells survived and there was considerable variation in colony size However, a second transfer to corn-steep-water agar resulted in very good growth, indicating that one transfer had sufficed to adapt the cells completely to this substrate The adaptation was lost completely by one transfer to malt agar The crucial test was made by showing that the cells also became adapted if allowed to stand one day in the cold room in corn-steep-water broth in the absence of cell division Luria and Delbrück's analysis is necessary if cells are increasing in number in the medium to which they become adapted, but if adaptation of a stationary population occurs, then their method is not necessary This fact alone proves that a heritable non-genic variation has been induced by an interaction of the substrate and cytoplasm which adapted the cells to growth in corn-steep water

The fact that heritable, induced adaptations (which should presumably be referred to the cytoplasm) occur makes it impossible, in microörganisms without a sexual cycle, to determine whether a given heritable variation is or is not a gene mutation The only possible technique for establishing the existence of a gene mutation is to make matings between the assumed mutant and a normal form and demonstrate that the genes controlling the expression of the characters segregate regularly at the reduction division New variations that are physiologically equivalent to gene mutations can be induced in bacteria by radiations In *Neurospora*, the genes responsible for synthesis of the B vitamins can be destroyed by radiation Roepke, Libby, and Small (62) showed that the ability of bacterial cells to synthesize B vitamins can also be destroyed by radiation and have suggested on the basis of the similarity of these experiments to those of Tatum and Beadle (73) that the new types which they obtained were bacterial gene mutations However, Lindegren and Lindegren (42) have shown that a large proportion of the heritable changes resulting from treatment of *Neurospora* with ultraviolet and X-rays are not gene mutations but some type of cytoplasmic change (Beadle and Tatum's experiments were devised in such a manner that this type of variant would not have been discovered) The production of cytoplasmic variants by radiation was demonstrated by mating different radiation-induced variants to normal individuals and finding that only normal progeny were obtained Since the progeny were haploid, a gene mutation would have given a 1 1 ratio of mutant to normal The fact that only normal progeny were obtained revealed that the genes in the radiation-induced variant were all normal, and the only possible explanation was that some constituents of the cytoplasm had been destroyed or injured, but that fusion with the cytoplasm of the untreated normal had replaced or repaired the deficiency

Induced Cytoplasmic Adaptation to Galactose

Kluyver (25) showed that *S cerevisiae* is capable of fermenting galactose, but the fermentation occurs only after a definite period of exposure to the sugar Spiegelman, Lindegren, and Hedgecock (70) found that when a suspension of diploid cells grown on glucose is washed with M/15 KH_2PO_4 and then resuspended in this solution with added purified galactose, the cell count remains constant over a long period of time When the addition of galactose is made in a Warburg vessel, fermentation begins explosively after a period of about three hours This action is an adaptation without the formation of new cells and under standard conditions the lag period is reproducible and is characteristic for the given strain Some strains have longer lag periods extending up to eight hours This phenomenon of acclimatization to galactose fermentation makes galactozymase in *S cerevisiae* an adaptive enzyme as defined by Karström (24) Since the adaptation occurs in the absence of cell division, it is due to the interaction of substrate with the cytoplasm, although it may nevertheless be basically under genetic control, as will be indicated later

Adaptation to Galactose by Mutation (?)

Spiegelman *et al* (70) tested a haplophase culture, derived from *S cerevisiae*, but incapable of fermenting galactose directly, for its capacity to adapt to galactose fermentation It was able to adapt to the fermentation of galactose after considerable, but variable, periods of growth in galactose broth An examination of the culture was made to determine if the starting population were homogeneous or heterogeneous Each cell was characterized by the type of colony which it produced when grown in agar under special conditions A cell suspension was plated on 4 per cent nutrient agar containing 4 per cent galactose and allowed to dry, then 5 per cent nutrient agar containing 4 per cent galactose was poured over the inoculated surface

Two kinds of colonies were observed one grew in the conventional lenticular form, appearing circular when observed from the top of the plate, the other produced an excessive amount of gas, resulting in cracks in the agar The former utilized galactose oxidatively with an R Q of 1, while the latter produced CO_2 in such excessive amounts that bubbles accumulated, rupturing the agar A large number of colonies was counted, revealing an average of about 7 per cent of fermenting type colonies with a variation in individual experiments from 2 to 15 per cent Similar test plates using glucose were examined and only one negative colony found in 2592 colonies examined The negative colony was examined manometrically and appeared to be a new non-fermentative variant

The technique was checked by making combinations of suspensions of cells The number of positive colonies appearing in the mixtures was the arithmetic mean of the percentages of positives in the two suspensions from which they originated, thus establishing the fact that local conditions in the plate had not induced fermentations

The percentages of positives obtained from positive colonies varied from 75 to 99 per cent, indicating that there was a selection of the fermentative type and that it tended to replace the non-fermentative type The non-fermenters used galactose slowly through aerobic oxidation and therefore their rate of division is depressed in this medium The few fermenters present, after a lag period, start to divide rapidly since they possess the enzymatic apparatus necessary to use this sugar at a rapid rate The number of fermenters thus increases due to two sources first, the rapid cell division of those already present, and second, the transformation of non-fermenting to fermenting cells This latter mechanism can, even with relatively low rates of change, be numerically significant in the early history of the populations because of the relatively large number of non-fermenters initially present On the other hand, the number of non-fermenters present at any time can increase only by virtue of simple cell division

A mathematical analysis by Spiegelman and Lindegren (68) revealed that when a glucose-grown haplophase culture was transferred to galactose broth the number of cells capable of producing fermenting colonies increased at a rate consistent with the hypothesis that natural selection had operated to enable the adapted to take precedence over the unadapted type That is, the ad-

vantage which the galactose-fermenter had over the galactose-oxidizer enabled the former to replace the latter This work was done before that of Luria and Delbrück (52) and the conclusion that the fermenters had arisen "spontaneously" rather than being "induced" by the presence of galactose was apparently unwarranted On this point our data on the haploid form were indeterminate

In contrast, the mathematical analysis applied to the diploid form revealed that the *explosive appearance of fermenters after the three-hour adaptation period could not possibly have arisen by growth and competition, but that essentially the whole population became adapted after the lag period* Therefore, the adaptation of the diplophase involves an interaction between the genetically-adaptable protoplasm and the substrate which results in the production of galactozymase by every cell

This presumably means that the adaptation of the non-fermenting haplophase involves *two steps* 1, *the transformation of* a genetically non-fermenting to a fermenting type (either spontaneously or by *induction) and* 2, a lag period in which the protoplast of the transformed type (the new "mutant," genetically like the adaptable diploid) reacts with the substrate galactose to produce galactozymase

The capacity of a protoplast to react with galactose to produce galactozymase is heritable, for the adaptable diplophase usually produces adaptable diplophases and the unadaptable haplophase usually produces unadaptables, a small percentage of which become transformed (either spontaneously or by induction) into adaptables This fact, however, does not establish it as a gene mutation as has already been indicated

Cytoplasmic Inheritance of Galactose-Fermenting Enzymes

S bayanus is incapable of fermenting galactose and a hybrid was made between it and *S cerevisiae* (Lindegren, 34) The diploid hybrid (*bayanus* × *cerevisiae*) culture was able to ferment galactose and the sixteen haploid *ascospore* cultures from four asci dissected from the hybrid were also capable of fermenting galactose One of this group of *first-generation ascospore* cultures was backcrossed to the original haplophase isolate of *S bayanus* and *the diploid* hybrid [(*bayanus* × *cerevisiae*) × *bayanus*] was capable of fermenting galactose Single ascospores were isolated from three asci and *all twelve* cultures were capable of fermenting galactose Subcultures obtained by plating some of these fermenting clones produced some *non-fermenting* cultures In view of our experiments on melibiose fermentation (see below), this particular experiment suggests that cytoplasmic factors have been involved which were transmitted to the progeny, independently of the germplasm, obscuring any Mendelian inheritance that may have been present *S bayanus* produces long, cylindrical, haploid cells, while *S cerevisiae* produces round, haploid cells The segregation of cylindrical versus round cells in the ascus was regularly Mendelian, indicating that a basic genic inheritance existed

Adaptation of Supposedly Unadaptable Yeasts

Schizosaccharomyces pombe, *Schizosaccharomyces octosporus*, and *Saccharomyces ludwigii* are among the non-fermenters of galactose All three yeasts were examined by Armstrong (1), who concluded that they were incapable of adaptation to galactose fermentation In *S ludwigii*, under ordinary conditions, the haplophase is transitory because fusion occurs within the ascus to reconstitute the diplophase almost immediately (fig 4) In *S octosporus* grown on solid medium the spores fuse very shortly after they are formed to reconstitute the diplophase In *S pombe*, the haplophase lasts a relatively longer time Adaptation of the species to galactose was studied by Spiegelman and Lindegren (59) Since mutations in the diplophase are not expressed unless they are dominant, it is not possible for selection mechanisms to operate to select them If one wishes to obtain extremely variable populations of yeasts this can be accomplished by inducing the diploid cells to sporulate and maintaining the culture in the haplophase since each mutation in the haplophase usually comes into immediate expression

The three species named above were induced to sporulate and inoculated into a medium containing 2 per cent glucose with 8 per cent galactose In from two to six days cultures of *S pombe* produced fermentation in the flasks and a stable galactose fermenter was obtained from the previously unadaptable form The glucose was necessary, apparently to insure sufficient initial growth to get a large population from which to select *S octosporus* and *S ludwigii* were not adaptable, presumably because in them the haplophase lasts a shorter time than in *S pombe*

S cerevisiae and *S carlsbergensis* are both capable of fermenting galactose, but non-fermenting haplophase variants have been obtained from both of these An additional non-fermenting haplophase culture was found in the examination of 2592 colonies of the galactose-fermenting haplophase of *S cerevisiae* described above, page 159 These three non-fermenters of galactose all used it aerobically with an R Q of 1 Microscopic examination revealed that they were haplophases When streaked on agar plates, they produced a great variety of colonial mutants, indicating that they had the genetical instability characteristic of haploid segregants However, they were all carried for five months on 8 per cent galactose without obtaining mutations to the fermentative type This seemed to indicate that some haploids may be quite incapable of the potentiality for mutating to produce galactose fermenters, although they may at the same time be quite unstable genetically and capable of producing a wide range of morphological variants

Genic Balance in the Haplophase

In the analysis of our hybridization experiments, we have found that all genes which control physiological reactions have proved to be dominant The heterozygote is capable of synthesizing a given vitamin just as well as the homozygote, and hybrids, heterozygous for the loci that control the fermentation of

melibiose, ferment melibiose just as well as homozygotes Therefore, mutations do not occur in the haplophase rather than in the diplophase, solely because the genes come into expression more easily in the haplophase, for dominant genes come into expression immediately in the diplophase

The phenomenon studied by Fisher (14), Wright (81) and Haldane (19) provides a possible explanation It has long been realized that naturally occurring genes are practically always dominant over mutant genes The genes controlling vitamin-syntheses and carbohydrate-fermentations are well established, naturally occurring genes that have probably become dominant by natural selection Newly mutated genes capable of inducing the fermentation of a sugar may be incapable of producing an effect in the heterozygous condition According to Fisher's theory, this would occur if all genes are originally recessive and become dominant against a specific genetical background He suggested that genes which have high survival value may become dominant through the selection of modifying genes in the evolution of the species By this mechanism a gene which was originally recessive becomes a stable dominant through a number of mutations at other loci Wright and Haldane suggest that modifiers may be relatively unimportant, but that the strength of the gene itself is increased by natural selection If Fisher's view be correct, the situation in yeasts may be explained as follows Yeasts are extremely heterozygous and may even be heterozygous for the fundamental modifiers that determine dominance If the balance of modifiers that control the dominance of many wild-type genes in the diplophase be upset when the haplophase is formed, the haplophase segregant would be more plastic than the original diplophase This may make it possible for mutations, which would ordinarily be suppressed by the complete complex of modifiers, to come into expression The mechanism making specific genes dominant is apparently much better stabilized in the case of genes controlling physiological activities than of those affecting the relatively less important morphological and colonial characters for the former are stable and dominant, while the latter are unstable and recessive In an interspecific heterozygote the gene controlling fermentation of a carbohydrate may be dominant simply because half of the genes in the heterozygote come from the parent species in which this gene has been evolved and therefore, half of the genome consists in part of modifiers, which act to make the fermenting gene dominant A mutation to ability to ferment galactose might fail to bring about the fermentation even if it occurred in the haplophase simply because a set of modifiers exists capable of suppressing this fermentation

The situation is further complicated since the apparent dominance of a fermentative mechanism may not be real, but may merely result from transfer of cytogenes Winge and Laustsen (79) found that in every case in which a fermenter was mated by a non-fermenter, the hybrid was capable of performing the fermentation To make certain that this is due to the dominance of the heterozygote, subsequent genetical analysis would be required Sometimes this analysis leads to confusion as in the pedigree of *S bayanus* × *S cerevisiae*, in this case cytoplasmic transfer of the cytogene is a complicating factor

Mendelian Inheritance of an Adaptive Enzyme

S cerevisiae is incapable of fermenting melibiose, and its haploid segregants fail to ferment this sugar even after continued growth in broth containing melibiose This suggests that the *S cerevisiae* genome limits the mutational range of this species, possibly by the mechanism discussed above *S carlsbergensis* is capable of fermenting melibiose, as are all its haploid segregants This is the principal character upon which *S cerevisiae* and *S carlsbergensis* are differentiated On the basis of the preceding discussion, the ability to ferment a single sugar may be sufficient to define two naturally occurring yeasts which produce viable four-spored asci Those cases in which the haplophases of one species are unable to adapt by mutation to the fermentation of a given sugar suggest that an elaborate system of modifiers may be necessary to enhance the expression of the gene in the other species Therefore, a single apparent genetical difference may mean that an elaborate hidden complex differentiates the two species However, as pointed out under "Speciation," fermentative differences in haplophase yeasts generally have little or no significance Figure 15 is a pedigree describing the progenies of matings between these two species (Lindegren, Spiegelman, and Lindegren, 50) The data were obtained by growing the cultures in a broth tube containing a smaller inverted tube to collect the gas produced by fermentation Accumulation of gas in the inverted tube is indicated by a plus sign

Hybrid I was an interspecific hybrid (*cerevisiae* × *carlsbergensis*) made by mixing melibiose-plus and melibiose-minus haplophase cultures Three diploid cells isolated after this mating were all capable of fermenting melibiose Eight asci were dissected from interspecific hybrids, and all the haplophase progeny were tested for the ability to ferment melibiose The results showed that all the haplophase cultures from three asci were melibiose +, two asci produced three + and one − culture, and one ascus produced two + and two − cultures

This experiment was performed before we had had any experience with the cytoplasmic transfer of enzymes and we thought that each haplophase culture which was able to ferment melibiose did so by virtue of a gene These data suggested that the haplophase of *S carlsbergensis* carried two genes capable of inducing the fermentation of melibiose However, a hybrid between the same *S carlsbergensis* haplophase and a haplophase incapable of fermenting either galactose or melibiose revealed that the *S carlsbergensis* haplophase carried only one gene controlling the fermentation of melibiose In this latter pedigree there was a 1 1 ratio in most of the asci for fermentation and non-fermentation of galactose as well as a 1 1 ratio for fementation and non-fermentation of melibiose, and these factors were segregated independently However, a few asci produced 4 spores, all of which fermented both melibiose and galactose The latter asci were clearly those in which a great deal of cytoplasmic transfer had occurred and the more frequent 1 1 ratio of mel + to mel − can be considered the proper Mendelian ratio unobscured by cytoplasmic transfer When transfer of the cytoplasm occurred, it included both enzymes as one would expect

Hybrid II was produced by backcrossing a positive haplophase culture from an ascus producing four + cultures with a negative haplophase culture from *S*

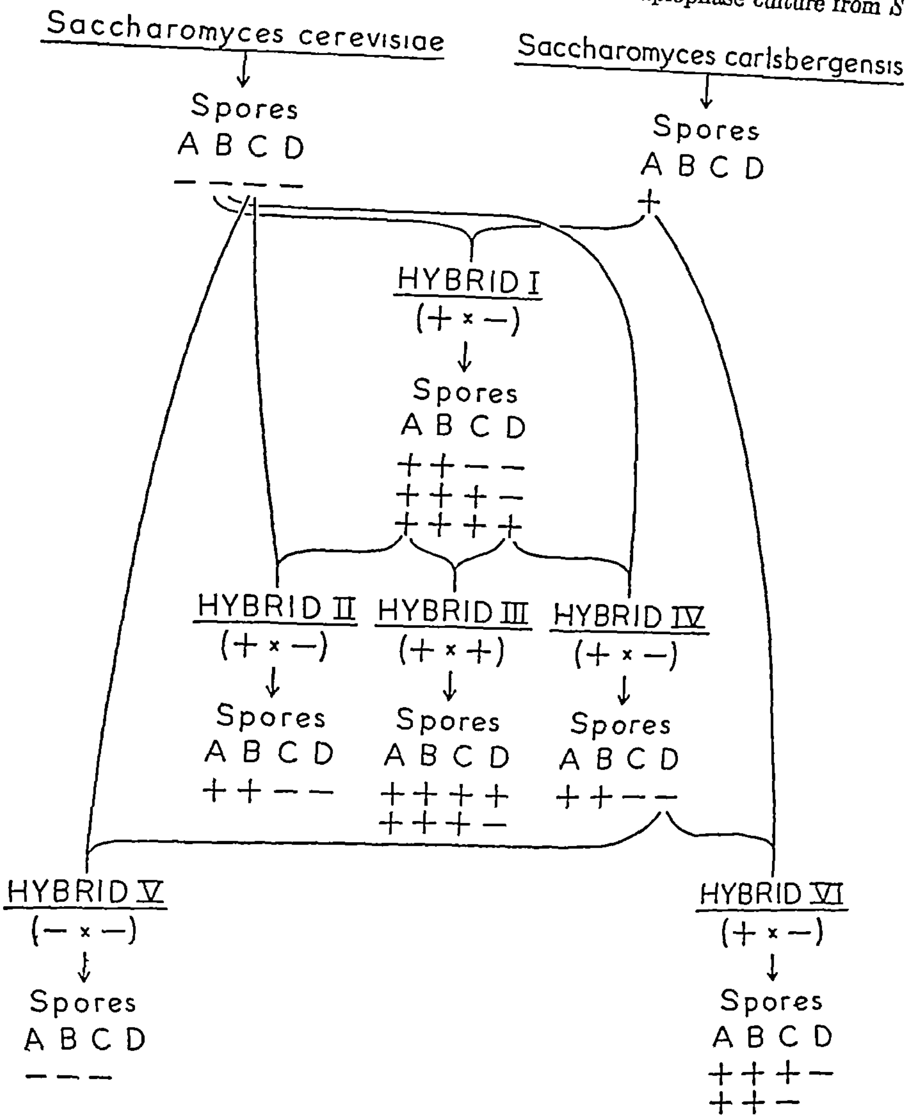

FIG 15 A pedigree showing the inheritance of an adaptive enzyme for the fermentation of melibiose *S cerevisiae* is incapable of fermenting the sugar while *S carlsbergensis* ferments it The diploid hybrid between these two ferments the sugar and produces asci from which single ascospores were isolated All four spores from some asci ferment the sugar Other asci contain one or two spores which fail to ferment the sugar Several other matings and backcrosses were made as indicated in the figure and discussed in greater detail in the text

cerevisiae Regular Mendelian segregation in the progeny shows that this haplophase carried a single gene, capable of controlling melibiose fermentation,

apparently derived from *S carlsbergensis* The backcross to *S cerevisiae* cleared up the Mendelian ratio by further dilution of the *S carlsbergensis* cytoplasm

Hybrid IV was made by backcrossing a second positive haplophase culture from the same ascus to a negative haplophase culture from *S cerevisiae* In this case, regular Mendelian segregation again shows this haplophase also carried a single melibiose-fermenting gene

Hybrid III was produced by mating the two positive cultures, each of which carried a single positive gene derived from *S carlsbergensis* Two of the twenty haplophase progeny failed to ferment melibiose Actually every one should have carried the gene, so these two failures may have been due to modifying genes from *S cerevisiae* suppressing the fermentation The inability of *S cerevisiae* haplophases to ferment melibiose after repeated trials suggests that such suppressors exist

Hybrid V was made by backcrossing a negative haplophase segregating from Hybrid IV to a negative haplophase from *S cerevisiae* The three haplophase progeny were all negative

Hybrid VI was made by backcrossing the same negative culture to a positive haplophase of *S carlsbergensis* Five of seven haplophase progeny fermented melibiose, while two failed

This pedigree is of especial interest because the fermentation of melibiose is due to an adaptive enzyme (Karström, 24) Cells which have been adapted to ferment melibiose lose this ability when removed from the substrate and have to be readapted to use it fermentatively

The Cytogene

In the preceding pedigree, the first contact with melibiose occurred when the culture was transferred to a fermentation tube containing melibiose A second series of experiments, (Spiegelman, Lindegren, and Lindegren, 71) showed that if contact with melibiose were maintained during the growth of the haplophase cultures during copulation, during growth on the pre-sporulation agar, and during spore formation, all four cultures obtained from a four-spored ascus of heterozygous diploids, such as Hybrids II and IV, were able to adapt to melibiose fermentation (Without this continued exposure to melibiose only two of the four cultures obtained from the four spores of each ascus are able to ferment melibiose and two from each ascus are unable to ferment melibiose) However, two of the four melibiose-fermenting cultures from each melibiose-treated ascus completely lost their ability to ferment melibiose when vigorously dissimilated by shaking in phosphate solution in the absence of melibiose This proves that melibiozymase was built up in the cytoplasm of the melibiose-fermenting gametes before copulation, maintained in the diploid hybrid and in the sporulating cell, and transmitted in the cytoplasm to each of the four spores irrespective of whether or not that particular spore carried the gene, and finally transmitted to the haplophase gametes derived from the spore even in the absence of the gene Melibiozymase was stabilized in the geneless clones containing a large amount of *S cerevisiae* genes and cytoplasm as long as melibiose

was present, but disappeared when the melibiose was withdrawn The melibiozymase was maintained in the cytoplasm solely by an interaction between melibiozymase and melibiose Therefore, melibiozymase is a self-perpetuating cytoplasmic entity which is gene-initiated, but whose quantitative level below a certain maximum depends on an interaction between melibiose and the enzyme and is independent of the gene so far as maintenance under these conditions is concerned

Genes initiate the production of the adaptive enzymes, but adaptation occurs only by interaction of the cytoplasm of the cells with the specific substrate, once the adaptive enzyme has been formed it is self-perpetuating in the presence of the substrate

I propose to call adaptive enzymes of this type *cytogenes* The fact that a period of exposure to melibiose must occur before melibiozymase is produced suggests that the original product is a relatively non-specific substance which is transformed into melibiozymase when it is "imprinted" by the melibiose The original relatively non-specific substance I propose to call the *protocytogene*

If the melibiose-plus gene transmits a protocytogene to the cytoplasm which becomes a specific cytogene by being "imprinted" by the melibiose molecule, it is possible that the same locus may be responsible for the production of other cytogenes as well The original gene-product which becomes specific by contact with the melibiose molecule might presumably become differently specific by contact with some other molecule Genes are "enzyme factories," but each gene may not necessarily be restricted to the production of a single enzyme

Sonneborn's (67) "killer" phenomenon can also be explained in terms of the following hypothesis based on our knowledge of cytogenes The *kappa* substance at present is a plasmagene which is stabilized by the *K* gene, but it originally was a cytogene produced by the (thus far undiscovered and hypothetical) *KAPPA* gene It has become established as a plasmagene by the transmission of the *kappa* substance from the cytoplasm of a heterozygous *KAPPA/kappa* individual to an individual carrying the *K* gene The *K* gene stabilized the *kappa* cytogene in a *kappa/kappa* individual and the cytogene was transformed into a plasmagene

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[Note added to Page Proof (Jan 17, 1946)]

Mendelian segregation of cytogenes In some recent experiments I made a hybrid between a clone carrying the recessive alleles for both melibiose and galactose fermentation by another with the recessive alleles but also carrying cytogenes cytoplasmically transmitted from the dominant The cytogenes were segregated in a one to one ratio at meiosis indicating that they could be carried on a recessive allele if it were contaminated with cytogenes This experiment shows that a locus on a chromosome is simply a passive place of attachment for cytogenes It confirms and extends Sonneborn's concept of the duality of the gene The gene does not generate anything except itself The dominant gene has a greater affinity for the cytogene than the recessive Many cytogenes reside at one locus moving into the cytoplasm when substrate appears and returning to the locus when the substrate has been transformed